

Mitochondrial Inheritance in *Ustilago violacea*

by

Gerhard A. A. Wilch, B.Sc. (Honours)

A Thesis

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements
for the Degree of
Master of Science

September, 1990
Brock University
St. Catharines, Ontario

© Gerhard A. A. Wilch

ABSTRACT

The anther smut fungus *Ustilago violacea* has been developed as an important model organism for genetic, morphological and physiological studies. Valuable information on the nuclear genetics on *Ustilago violacea* has been obtained in the last 20-25 years. However, in this organism almost nothing is known about mitochondria which make up an important aspect of the fungal genetic system. One fundamental aspect, mitochondrial inheritance, was addressed by this investigation.

Mitochondrial DNA (mtDNA) of *U. violacea* was purified and restriction fragments cloned. MtDNA restriction fragment length polymorphisms (RFLPs) were identified among different isolates and were used as genetic markers for studying mitochondrial inheritance in crosses between polymorphic isolates. Matings of the yeast-like haploid cells of opposite mating types resulted in dikaryons containing mitochondria from both parents. The dikaryons were induced to form hyphae and then allowed to revert to haploid growth, resulting in a colony that is bisected for the two nuclear types. Both nuclear-type progeny of each cross were examined for parental mitochondrial type: Either mitochondrial type was observed in the progeny. Thus, mitochondrial inheritance is biparental in this organism. The recovery of both mitochondrial types in the progeny was non-random. In progeny with the nuclear genotype of the a_1 mating type parent mitochondria from both parents were inherited equally well. However, in progeny with the a_2 mating type, mitochondria were inherited almost exclusively (94%) from the a_2 parent.

ACKNOWLEDGEMENTS

For the opportunity to begin the exciting new work on mitochondrial inheritance in *Ustilago violacea*, I am very grateful to Dr. A. J. Castle, and I wish to express my sincere appreciation for his excellent supervision, and the support and good example he provided.

In addition I would like to thank the members who served on my advisory committee, Dr. D. J. Ursino, Dr. Y. Haj-Ahmad, and Dr. W. H. Cade for their guidance and helpful suggestions.

I am indebted to Dr. R. L. Carlone for giving valuable advice and sharing his lab. Thanks also to Dianne Eaton for taking care of the plants in the greenhouse, Marion Vigh, Rob Boulianne, Mitzi Seifried and Paula Argenta for their friendship and help in the lab.

I would also like to add that the wonderful cooperation of all the faculty and staff helped make this a memorable learning experience.

Finally, I thank my parents for their loving support and direction which made this all possible.

TABLE OF CONTENTS

ABSTRACT	2
ACKNOWLEDGEMENTS	3
TABLE OF CONTENTS	4
LIST OF TABLES	6
LIST OF FIGURES	7
1. INTRODUCTION	8
1.1 Ojectives	8
1.2 Mitochondrial Genomes	10
1.2.1 Mitochondrial DNA Size and Copy Number	10
1.2.2 Mitochondrial Encoded Genes	12
1.3 Mitochondrial Genetic Markers	12
1.4 Mitochondrial Inheritance	14
1.4.1 Uniparental Inheritance	15
1.4.2 Biparental Inheritance	16
1.4.3 Generation of Homoplasmic Cells	17
1.4.4 Inheritance in Basidiomycetes	18
1.5 Life Cycle of <i>Ustilago violacea</i>	20
2. MATERIALS AND METHODS	23
2.1 Collection of Isolates	23
2.2 Germination and Growth of Cultures	23
2.3 Techniques for DNA Preparations.....	26
2.3.1 Breaking the Cell Wall	26
2.3.2 Mitochondrial DNA Isolation	27
2.3.3 Small-Scale Total DNA Isolation	34
2.4 DNA Analysis Techniques	35

2.4.1 Restriction Endonuclease Digestion	3 5
2.4.2 Agarose-Gel Electrophoresis	3 5
2.4.3 Southern Blotting	3 6
2.4.4 Radioactive Labelling of DNA Probes	3 7
2.4.5 Hybridization of Southern Blot with Labeled DNA	3 8
2.5 Cloning of Mitochondrial DNA Fragments	3 9
2.5.1 Plasmid Preparation	3 9
2.5.2 Ligation Treatment	4 0
2.5.3 Transformation of <i>E. coli</i> with pUC 13 Plasmid	4 0
2.5.4 Analysis of Clones	4 5
2.6 Determining <i>U. violacea</i> Isolates Polymorphic in MtDNA	4 7
2.7 Crosses	4 7
3. RESULTS	4 9
3.1 Estimate of Mitochondrial Genome Size	4 9
3.2 Mitochondrial Probe Construction	5 4
3.3 RFLP Analysis	5 7
3.3.1 Mitochondrial Markers	5 7
3.3.2 Performing Crosses	5 7
3.4 Analysis of Results	6 3
3.4.1 Isolate Differences	6 5
3.4.2 Mating Type Differences	6 5
3.4.3 Transfer of Mitochondria through the Conjugation Tube	6 8
4. DISCUSSION	7 8
5. LITERATURE CITED	9 0

LIST OF TABLES

Table	page
1. <i>U. violacea</i> Isolates Used in this Study	24
2. UWO-1 MtDNA Fragments Generated with <i>Eco</i> R1 Digestion and the Corresponding Clones that were Obtained	53
3. Restriction Fragment Sizes of <i>Eco</i> R1 Digested MtDNAs from Different <i>U. violacea</i> Isolates.....	58
4. Mitochondrial Types, According to Parent Type, of Progeny from Crosses Between Isolates of <i>U. violacea</i>	60
5. Analysis of Nuclear and Mitochondrial Genotypes from Bisected Colonies	64
6. Recovery of Progeny with the same Mitochondrial Genotype as the Parent Isolate	66
7. Parental Origin of the Mitochondria in a_1 and a_2 Progeny and Combined Results in Crosses of UWO-1 with BU-1 and BU-3	67
8. Analysis of Hyphal Formation from Various Crosses Involving Young and Old Cultures	61
9. Bidirectional Model: Comparison with Results Obtained	75
10. Unidirectional Model: Comparison with Results Obtained	76

LIST OF FIGURES

Figure	Page
1. Life Cycle Diagram of <i>Ustilago violacea</i>	2 1
2. DNA Isolation Techniques:	
A. MtDNA Isolation	3 1
B. Total DNA Isolation	3 2
C. DNA Analysis	3 3
3. MtDNA Probe Construction:	
A. DNA and Plasmid Preparation	4 3
B. Transformation and Analysis	4 4
4. Agarose-Gel Electrophoresis of Mitochondrial and Nuclear DNA Digested with Three Different Restriction Enzymes	5 2
5. Hybridization of a Nuclear Clone Containing Putative rDNA Sequences to a Blot of <i>Eco</i> R1 Digested Nuclear and MtDNAs	5 6
6. The Presence of Both Parental Mitochondrial Types in DNA Samples Derived From Single Cells	6 2
7. Models of Inheritance:	
A. Bidirectional Transfer	7 3
B. Unidirectional Transfer	7 4

INTRODUCTION

1.1 Objectives

Members of the Ustilaginales have proven very useful for genetic research due to the convenience provided by a readily cultured haploid phase of their life cycle. This advantage has led to the collection of valuable information about various genetic mechanisms. For example, substantial insight into molecular recombination and gene conversion was obtained in studies with the corn smut fungus *Ustilago maydis* (Holliday 1961, 1964).

The anther smut fungus *Ustilago violacea* has a wide host range, infecting 70 members of the Caryophyllaceae (Zillig 1921, Liro 1924). Initial investigations into the genetics of this organism (Day and Jones 1968, 1969) demonstrated the usefulness of *Ustilago violacea* for further studies into the biology of this plant pathogen. The nuclear genetics of *Ustilago violacea* have been well characterized (Castle and Day 1980, 1981, Catterall *et al.* 1978, Cummins and Day 1973, Day 1972, 1978, 1979, Day and Day 1974, Day and Jones 1969, 1971, Garber and Day 1985, Garber *et al.* 1983). Very little, however, is known about the inheritance of mitochondria. This is of particular interest, since the mechanism of organelle transmission is always distinct from that of genes in the nucleus.

The aim of the present study was to examine the type of mitochondrial inheritance manifested by *U. violacea*. This was accomplished by identifying mtDNA restriction fragment length polymorphisms (RFLPs) among different isolates and using the RFLPs as mitochondrial markers for tracking inheritance in the progeny of crosses. Mitochondrial inheritance was investigated by mating haploid

saprophytic sporidia which contain different mitochondrial RFLP markers. Formation of the dikaryon was followed by induction of hyphal growth with vitamin E (Castle and Day 1984). This process is analogous to the formation of infection hyphae on host plant tissue (Day *et al.* 1981, Day and Castle 1982). The progeny issuing from such crosses were then analyzed for the mitochondria that they had inherited by comparing them with the parental mitochondrial types.

1.2 Mitochondrial Genomes

The mitochondrial genome is an important aspect of an organism's genetic system due to the central role of mitochondrial respiration in cellular metabolism, growth and development. The importance of this is evident in the effects mutant mitochondria can have on an organism. In humans a broad spectrum of neuromuscular diseases has been associated with alterations in mitochondrial genes encoding functions required for oxidative phosphorylation. Deleterious effects of these mutations are more pronounced in tissues that rely heavily on mitochondrial energy: the central nervous system, skeletal muscle, heart, kidney and liver (reviewed by Wallace 1989). The following sections briefly review pertinent aspects of mitochondrial DNAs.

1.2.1 Mitochondrial DNA Size and Copy Number

The properties of mitochondrial genome size and arrangement are very different among the different kingdoms: In the plants, for example, the mitochondrial genomes studied were found to range in size from 200-2500 kilobases (kb) (reviewed by Palmer 1990). In contrast, animal mtDNAs are strikingly uniform in structure, contain almost no non coding sequences, and are very small, about 16-19 kb (Avisé and Lansman 1983). The mtDNAs of fungi show a high degree of variation, ranging in size from 18.9 kb in *Torulopsis glabrata* (Clark-Walker and Sriprakash 1981) to over 176 kb in *Agaricus bitorquis* (Hintz *et al.* 1985). A large variability may even exist between different species belonging to the same genus. For example, in *Agaricus brunnescens*, the mitochondrial genome is only 56% as large as that of *A. bitorquis*. A twofold size difference was also observed among two species of *Coprinus* (Weber *et al.* 1986). Similarly, within *Saccharomyces*, mtDNA sizes

ranged from 23.7 kb in *S. exiguus* to 78 kb in *S. cerevisiae* (Clark-Walker *et al.* 1983). Large interspecific mitochondrial genome size variations are also present in plants. In the family Cucurbitaceae an 8-fold size difference was observed (Ward *et al.* 1981). The difference between the size of animal, plant and fungal mtDNAs, and the variability among plant and fungal mtDNAs is apparently due to optional introns (animal mitochondria are free of introns) and deletions and insertions of spacer regions (Taylor 1986).

The mitochondrial genetics of *Saccharomyces cerevisiae* has been well studied and is the best understood of any organism: *S. cerevisiae* has between 10 mitochondria/cell in rapidly growing cells and 50 mitochondria/cell in stationary phase cells. Rapidly growing cells usually contain one mitochondrion that is much larger than the others, encompassing as much as 50% of the total cell's chondriome (Stevens 1977). In yeast mitochondria are usually always in a mobile and flexible state with branches forming, pinching off, fusing together and changing location (Williamson 1976, *cf* Dujon 1981). Each organelle contains from 1 to 20 mtDNA molecules, with as much as 100 copies present in a cell. MtDNA molecules within the mitochondrion are not randomly distributed, but are normally confined to small DNA regions, of which about 10-40 regions are visible under the microscope when stained for DNA (Dujon and Slonimski 1976). Recombination between mtDNA molecules has been demonstrated to occur at a very high frequency, which might be expected from the highly mobile nature of the mitochondrion (Fonty *et al.* 1978).

1.2.2 Mitochondrial Encoded Genes

In spite of the great superficial differences in size and arrangement between animal, plant and fungal mitochondrial genomes, they all contain nearly the same genes, coding for enzymes or their subunits involved in electron transport and phosphorylation to produce ATP. These include cytochrome *c* oxidase, apocytochrome *b* and two subunits of the ATPase. Other genes encode mitochondrial RNAs and proteins required for protein synthesis like the *var-1* ribosomal protein, 5-S ribosomal protein, S- and L-rRNA, and a number of tRNAs. Included are also a few unidentified open reading frames (Grossman and Hudspeth 1985).

1.3 Mitochondrial Genetic Markers

In order to study mitochondrial inheritance, markers are necessary to identify parental mitochondria. Naturally occurring alterations of mtDNA have been described for many eukaryotic organisms and have been correlated with the well-known phenomenon of respiratory deficiency in the yeast *Saccharomyces cerevisiae* (Ephrussi *et al.* 1955), male sterility in some higher plants (Edwardson 1970) and aging or vegetative decline in the Ascomycete fungi *Podospora anserina* (Kuck and Esser 1982), *Neurospora crassa* (Rieck *et al.* 1982, Griffiths and Bertrand 1984) and *Aspergillus amstelodami* (reviewed by Esser *et al.* 1986). In *Saccharomyces cerevisiae*, spontaneous mtDNA mutants arise as a result of erroneous mitochondrial recombination. These mutant mitochondria outcompete by some unknown mechanism the normal mtDNAs, resulting in cultures homoplasmic for the mutant mitochondrial allele that are unable to respire (Backer and Birky 1985). *Neurospora* strains from most wild

sources could potentially grow indefinitely. *N. intermedia* and a *N. crassa* strains have been isolated that show a limited duration of vegetative growth (Rieck *et al.* 1982; Griffiths and Bertrand 1984). Insertion of a linear plasmid in the mitochondrial chromosome and the subsequent accumulation of altered mitochondria leads to respiratory deficiency, aging and cell death. A plasmid termed *kalilo* has been identified as the responsible element for this type of senescence (Bertrand *et al.* 1985).

Many conventional techniques for genetically marking nuclear DNAs do not work with mitochondria since the latter involve a multi-copy genetic system. Special selective pressures are required for sorting out a particular mutated mtDNA molecule from the rest of the population. The majority of information on cellular and molecular mechanisms of organelle transmission genetics has been obtained from *Saccharomyces cerevisiae* and *Chlamydomonas reinhardtii* for which a number of mitochondrial markers were available permitting a detailed analysis. The first mitochondrial mutants characterized were the spontaneously forming *rho*⁻ mutants of *S. cerevisiae*. These lack mtDNAs and the mitochondria are nonfunctional, with the cells unable to grow on nonfermentable medium. The *rho*⁻ mutants can also be induced in very high frequency using a number of chemicals, *e.g.* ethidium bromide (Dujon 1981). Mitochondrially inherited respiratory-deficient mutants other than *rho*⁻ were obtained using Mn⁺⁺ mutagenesis. This method causes errors in mtDNA due to defective mtDNA replication resulting in point mutations and other mutations useful for studying the functions of the mitochondrial proteins (Putrament *et al.* 1975).

Most other organisms are unable to survive without respiration, making it impossible to use such mutations for studying mitochondrial

inheritance. Other mutations which confer antibiotic resistance to mitochondria, like the *ery^R* mutation, resulting in erythromycin resistance can be used, however, this method still involves considerable difficulty.

In the absence of mitochondrial genetic markers, naturally occurring polymorphisms in fungal mtDNAs are currently being developed as genetic markers for studying cytoplasmic inheritance and gaining phylogenetic information about a broad range of fungal species. Length mutations, which are common in most fungal mtDNAs can be used as markers between two isolates or species. MtDNA RFLPs have been found to occur in high frequency among and within fungal species, including *Agaricus bitorquis* (Hintz *et al.* 1985) and *Schizophyllum commune* (Specht *et al.* 1983). Mitochondrial RFLP markers are easy to generate and are an excellent system for studying mtDNA transmission in virtually any organism where there are no other markers available. Using multiple RFLP markers, it is also possible to detect recombination events. RFLP mapping is relatively easily done, although it can be time consuming.

1.4 Mitochondrial Inheritance

The first evidence for organelle heredity was presented already 80 years ago by Correns in 1909 and Baur in the same year (*cf.* Dujon 1981) when they discovered that some genetic characters, affecting the plastid phenotype of higher plants, show a non-Mendelian type of inheritance. Non-Mendelian organelle heredity was first demonstrated in fungi by Ephrussi *et al.* (1949, *cf.* Dujon 1981) who studied the respiratory deficient mutants of *Saccharomyces cerevisiae* induced by acriflavin.

Unlike animal and most plant systems, various modes of mitochondrial inheritance are known to exist among fungi. One of these is uniparental inheritance, which may be due to the failure of gametes from one parent to transmit mitochondria to the zygote, *e.g.* monogametic organelle transmission (Mogensen 1988), or due to the transmission of few mitochondria from one parent in relation to the other, *e.g.*, in the case of sperm and egg (for review see Birky 1978). Biparental inheritance of mitochondria is the event in which both parents are able to donate mitochondria to offspring. This, however, does not necessitate the formation of mixed populations of mitochondria (May and Taylor 1988). When mixed populations are formed, genetic recombination between parental molecules is possible (Birky 1978). Examples of these different modes of inheritance in fungi are given below.

1.4.1 Uniparental Inheritance

Uniparental mitochondrial inheritance is a type of transmission pattern in which mitochondria are inherited from only one parent. This has been reported in *Neurospora crassa* where the larger gamete transmits mitochondria to the offspring (Mitchell and Mitchell 1952, Manella *et al.* 1979). This is also known as maternal inheritance and is analogous to mitochondrial inheritance in animals and most plants. Maternal inheritance of mitochondria is usually a result of the very disproportionate contribution of mtDNA molecules from one of the two parents. Paternally inherited mitochondria, in which the smaller, more active gamete contributes mitochondria, was found to be the case in interspecific crosses of *Allomyces macrogynus* with *A. arbusculus* (Borkhardt and Olson 1983).

Many fungi have gametes that are morphologically indistinguishable so that they cannot be referred to as male or female. Instead, they are referred to as compatible or incompatible mating types. Among closely related strains of the cellular slime mold *Polysphondylium pallidum* mitochondrial inheritance is uniparental with one mating type (*mat 2*) being dominant to the other with respect to the mtDNA transmission pattern. This relationship, however, breaks down when more distantly related strains are mated, resulting in the transmission of both mitochondrial types (Mirfakhrai *et al.* 1990). In *Physarum polycephalum*, mitochondrial transmission has been shown to be regulated by multiple alleles of the *mat A* locus, which form a hierarchy with respect to mitochondrial transmission (Kawano and Kuroiwa 1989).

1.4.2 Biparental Inheritance

Biparental mitochondrial inheritance in which mitochondria are inherited from both gametes has been shown in the plasmodial slime mold *Didymium iridis* (Silliker 1985) and in the yeast *Saccharomyces cerevisiae* (Borst and Grivell 1978). When mitochondria from both parental gametes are transmitted to daughter cells one might expect the potential for extensive interaction between mtDNA molecules. This has been demonstrated in *S. cerevisiae* where recombination has been detected in the zygote (Fonty *et al.* 1978). Mitochondrial inheritance in basidiomycetes studied to date was also found to be biparental. These are discussed in detail below in section 1.4.4.

1.4.3 Generation of Homoplasmic Cells

Although cytoplasmic particles are contributed equally from both parents in *Saccharomyces cerevisiae*, few progeny remain heteroplasmic after 10-20 generations. The trend towards the generation of homoplasmic cells is probably mostly due to a biased input of mtDNA molecules into the buds. Many zygotes transmit the alleles which are present in the majority (Birky 1978). If mixing of contents is not rapid and complete, early buds may contain strongly biased amounts of parental mtDNA molecules. Buds emanating from the ends are more likely to be biased than those from the center of the zygote (Strausberg and Perlman 1978). Extensive recombination and genetic drift - random, unequal replication of mitochondrial genomes - also contributes to the rapid generation of homoplasmic cells (Birky 1978). Recombinants, since they are unique and few in number, are usually diluted out by genetic drift. An exception to this rule are the petites of *S. cerevisiae*.

Suppressive petites originate as a result of a cell becoming homoplasmic for a mitochondrial mutation which is able to replace (suppress) the wild type phenotype. Since this type of mutation involves the deletion of large portions of the mtDNA molecule by recombination, the resulting culture is unable to respire. Study of these mutants has revealed that enhanced transmission of the petite genome may be due to the replicative superiority of the mutant mtDNA sequences (Carnevali 1969) and/or enhanced segregational efficiency into buds (Chambers and Gingold 1986). Various origin of replication (ori) sequences were found to be present in greater proportion in the suppressive petites relative to the wild type cells (Bernardi *et al.* 1980, Blanc and Dujon 1980). These were found to be responsible for

positively influencing transmission of mtDNA into daughter cells (Piskur 1988). The analysis of three mutants lacking *oril* revealed that those mutants which had larger deletions encompassing more of the surrounding intergenic sequences were more deficient in transmission, suggesting that these sequences may also be important in transmission.

1.4.4 Inheritance in Basidiomycetes

Within the basidiomycetes, to which *Ustilago violacea* belongs, mitochondrial inheritance has been found to be biparental in all cases studied. Mitochondrial inheritance has been studied in a few members in which mating occurs in filamentous cultures. In *Coprinus cinereus* mating occurs by anastomosis between uninucleate hyphae. In compatible matings, nuclei are exchanged and migrate throughout the partner's mycelia, resulting in dikaryons. Mitochondria, however, are not transmitted into the partner's mycelium. Therefore both mated cultures become identical for nuclear character but retain their original mitochondrial types (Casselton and Condit 1972, Baptista-Ferreira *et al.* 1983). Similar results were obtained with *Armillaria mellea* (M. L. Smith and J. B. Anderson, personal communication). In the anastomosed cells, mixed mitochondrial types do occur. Economou *et al.* (1986) have detected mitochondrial recombination at the mating junction in *Coprinus cinereus*.

Agaricus bitorquis, another basidiomycete which mates by hyphal fusion, rarely exhibits nuclear migration. Instead, nuclear exchange and mitochondrial mixing only occurs in the junction of mated cultures and from there hyphae may grow out. In one case, unilateral nuclear migration from a rare nuclear donor strain to a recipient was observed. Here mitochondria did not migrate with the nuclei (Hintz *et al.* 1988).

Mitochondrial inheritance in basidiomycetous fungi in which unicellular gametes mate, as opposed to anastomosis between vegetative hyphae, has not yet been studied. Therefore this study into mitochondrial inheritance is very timely in beginning to understand the transmission of mitochondria in these important members of the basidiomycetes.

In order to study mitochondrial inheritance in *Ustilago violacea*, strains with known RFLPs can be crossed and their progeny analyzed for mitochondrial type. *U. violacea* is a good candidate for determining mitochondrial inheritance, since it has a well-defined life cycle. Since various modes of mitochondrial inheritance are known to operate among fungi, no *a priori* assumptions about mitochondrial inheritance can be made about *Ustilago violacea*.

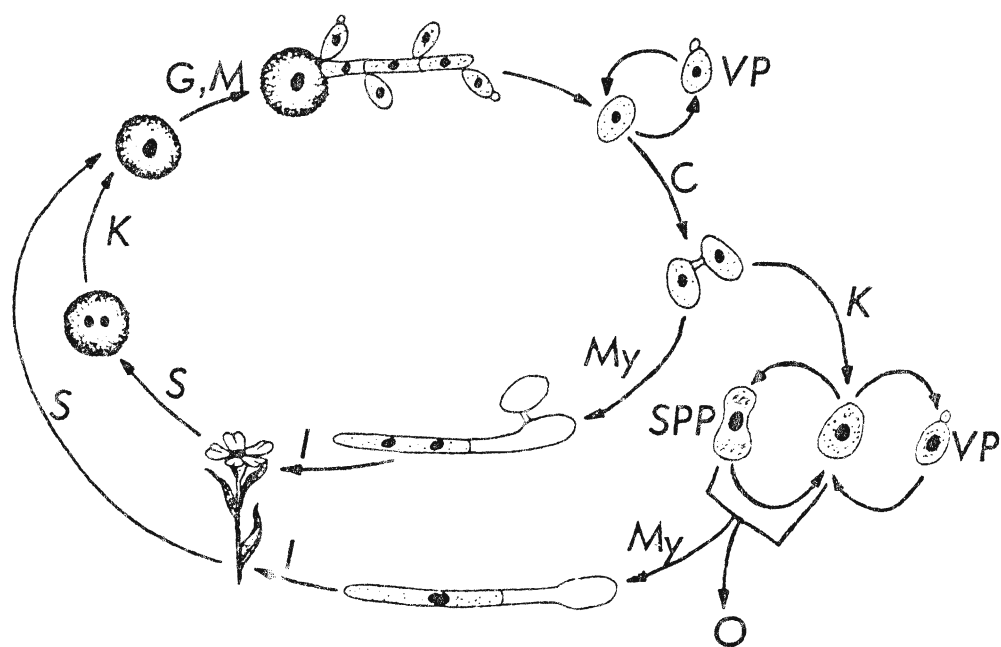
1.5 Life Cycle of *Ustilago violacea*

Over 70 species of the Carnation family, Caryophyllaceae, are susceptible to infection by *U. violacea* (Zillig 1921; Liro 1924). *Silene alba*, known commonly as White Champion, is the host plant for the isolates on which this work is based. The life cycle of *U. violacea* (Fig. 1), as described in Fisher and Holton (1957) and modified by Castle and Day (1980), consists of a saprophytic and a parasitic state. Diploid teliospores taken from the anthers of an infected host produce a three-celled promycelium upon germination on a moist surface. Meiosis occurs during germination resulting in four haploid products which bud off as yeast-like cells from the teliospore and the three cells of the promycelium. On nutritive medium the saprophytic sporidia undergo continued cellular division in the absence of the host plant. The sporidia are of two mating types, determined by alleles a_1 and a_2 of the mating

Figure 1: Life Cycle of *Ustilago violacea*.

Legend: G - teliospore germination
M - meiosis
VP - vegetative phase
C - conjugation
K - karyogamy
SPP - sexual spore precursor
My - mycelium formation
I - infection
S - teliospore formation
O - opaque strain formation

(Based upon Fisher and Holton, 1957; Castle and Day, 1980)



locus. On the surface of a host plant or non-nutritive medium, conjugation of compatible sporidia (a_1 with a_2) occurs. The a_2 cells produce conjugation pegs which grow out as conjugation tubes toward a_1 cells and a cytoplasmic connection is made (Poon and Day 1974). On the host surface, conjugation results in a dikaryon from which an infection hypha forms from one of the mated cells. Following penetration of the host plant, the dikaryotic hypha continues to grow intercellularly, infecting the whole plant. When the flower buds begin to form, teliospore formation begins in the developing anthers, replacing pollen (Batcho *et al.* 1979). When sporulation is complete the dikaryotic teliospores undergo karyogamy.

The addition of vitamin E to a culture of mated dikaryons on non-nutritive medium will also result in the formation of hyphae that are analogous to the infection hyphae on the host plant surface (Castle and Day 1984). As the mycelium grows out from the sporidium, both nuclei and cytoplasm follow the advancing hyphal tip. After exhaustion and/or oxidation of the vitamin E, haploid, yeast-like cells bud off from the hypha on nutritive medium, and continued mitotic budding results in the formation of a colony in which both parental nuclear genotypes are present. What happens to the original parental mitochondria following such a cross was still unknown up to this point and it was the purpose of this thesis to shed some light on this fundamental question.

MATERIALS AND METHODS

2.1 Collection of Isolates

Strains used were derived from different isolates of *Ustilago violacea*, from the host plant *Silene alba*, White Campion (Table 1). Some isolates (designated UWO-1 to -27, mostly from the U. K.) were from the collection of A. Day. Other isolates (designated BU-1 to -6) were collected by myself in Germany and Hungary. In obtaining isolates, plants were first identified and checked for infection with *U. violacea*, which was apparent by the purple color due to teliospore production in the anthers. Uninfected plants have yellow anthers. Immature (closed) flower buds were collected from infected plants and kept on silica gel.

2.2 Germination and Maintenance of Cultures

Teliospores from infected anthers were germinated on complete medium (CM, see below) and the culture was streak-plated to obtain single colonies. Strains were subcultured on CM, and after growing three to five days at room temperature, they were transferred to a refrigerator (4°C) and every month, the strains were subcultured. Actively growing cultures were obtained by inoculating the desired medium with a sample from the cold storage plates.

Culture Conditions

The media outlined here were developed by A. W. Day and J. K. Jones (1968). These media are useful for the culture of the sporidial phase of the life cycle.

Table 1. *Ustilago violacea* Isolates Used in this Study

Collection	Date	Site	Host Plant	Collector
UWO-1	1964-65	Reading, UK	Silene alba	D. Snow
UWO-3	14/06/75	Goodwood, UK	S. alba	A. Day
UWO-25	5/08/79	Doncaster, UK	S. alba	A. Day
UWO-27	-	Abingdon, UK	S. alba	G. Kirby
BU-1	23/06/88	Tiergarten, Berlin	S. alba	G. Wilch
BU-3	6/07/88	Ruhleben, Berlin	S. alba	G. Wilch
BU-4	12/07/88	Grunewald, Berlin	S. alba	G. Wilch
BU-5	21/07/88	Hortobagy-1, Hungary	S. alba	G. Wilch
BU-6	21/07/88	Hortobagy-2, Hungary	S. alba	G. Wilch

Media

Water Agar (WA)

Agar	20-25 grams
Distilled Water	To 1 liter

Complete Medium (CM)

Glucose	10 grams
Peptone	10 grams
Yeast Extract	3 grams
Malt Extract	3 grams
Distilled Water	To 1 liter
Agar (for plates)	20 grams

(All components of the media were obtained from DIFCO Laboratories, Detroit MI.)

Mating Tests

Conjugation between haploids of opposite mating type is induced by both low temperature and low nutrient conditions. The recovery of mated dikaryons is greatest after a 24-48 h incubation period at 15°C on water agar (Day and Jones 1968). These conditions were used to determine the mating type of each strain. Five single colonies on a streak plate of germinated teliospores were tested for mating type. Samples of these colonies were mixed with samples of known a_1 and a_2 cultures. The next day the mixed cultures were screened microscopically for the presence of conjugation tubes between cells. Since mating occurs only between cells expressing opposite mating types, the presence of conjugated cells means that the unknown must express the mating type allele opposite to the known strain.

Growth and Harvesting of Cultures

Cultures were grown in 100 to 500 ml of liquid CM in flasks that were vigorously agitated at room temperature (RT) for three to four

days. Before harvesting, a sample from each culture was examined under the microscope to determine the purity of the culture. Uncontaminated cultures were harvested in 250 ml centrifuge bottles which were centrifuged five minutes at 2000 x g. The cell pellet was washed with sterile water, recentrifuged and suspended in distilled water.

The concentrated cell suspensions were frozen in liquid nitrogen in order to disrupt the cells. The frozen cells were lyophilized, and stored at -20°C.

2.3 Techniques for DNA Preparations

Chemicals used were obtained from Sigma Chemical Co., St. Louis, MO, Fisher Scientific Co., Fairlawn, NJ; Pharmacia LKB, Biotechnology AB, Uppsala, Sweden and Bio-Rad Laboratories, Richmond, Calif. Enzymes were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN. and Bethesda Research Laboratories Canada, Burlington, Ontario (unless indicated otherwise), and [α -³²P]dCTP was obtained from ICN, Irvine, Calif.

2.3.1 Cell Wall Breakage

A number of difficulties with the extraction of DNA from *U. violacea* cells were encountered and several techniques were developed to overcome these problems. Microscopic examination of freeze-dried cells revealed that less than 0.1% of the cells showed signs of disruption. Since *U. violacea* cells have a tough cell wall, extraction of DNA was quite inefficient. To date no commercially available enzymes are known to degrade the cell wall of *U. violacea*. Two different methods were attempted to generate a higher DNA yield: Sonication and physical

abrasion. Sonication did not result in an improvement in the yield of DNA. Alternatively, cells were mixed with silica gel as an abrasive and were ground with a mortar and pestle. Grinding for four minutes in TE (50 mM Tris base (adjusted to pH 8.0 with HCl), 10 mM ethylenediaminetetraacetic acid (EDTA) at RT resulted in a 10-fold increase in yield, but the DNA appeared very degraded, probably due to extensive shearing. Briefly grinding cells in liquid nitrogen for 30 seconds resulted in good-quality DNA and a modest increase in yield. Since pure, high molecular weight DNA was required for cloning, the latter method was adopted.

2.3.2 Mitochondrial DNA Isolation (Fig. 2A)

Variations of the DNA isolation techniques published by Murray and Thompson (1980) were employed for this experiment.

Extraction buffer. For 0.5-1.0 g of freeze dried *U. violacea* cells, 10 ml of extraction buffer was prepared consisting of 50 mM Tris (pH 8.0), 0.7 M NaCl, 1.0% w:v hexadecyltrimethylammonium bromide (CTAB), 10 mM EDTA, 1.0% (v:v) 2-mercaptoethanol.

The extraction buffer and cells were mixed together in a 30 ml Corex glass centrifuge tube, and with the aid of a glass rod lumps were gently dispersed. The mixture was incubated for 25-45 minutes at 55°C with occasional gentle mixing.

An equal volume of chloroform : isoamyl alcohol (24:1) was added and emulsified by very gentle inversion. The tube was centrifuged (13,000 x g, 10 min., 25°C, SS-34 rotor) and three phases were observed: a lower organic phase, a middle debris layer and an upper aqueous layer containing the nucleic acids. The upper aqueous layer was transferred to a clean centrifuge tube. One tenth volume of high CTAB-high salt

(1.0% CTAB, w:v, in 0.7 M NaCl) was added to the aqueous phase and the chloroform separation was repeated.

Nucleic acid recovery. The nucleic acids were precipitated by reducing the NaCl concentration from 0.7 to 0.35 M by adding one volume of CTAB-no salt buffer (1.0% CTAB, 50 mM Tris-HCl pH 8.0 and 10 mM EDTA) and incubating for 30 min. at RT. The precipitate was recovered by centrifugation (2000 x g, 5 min., 25°C, SS-34 rotor).

Cesium chloride gradient extraction. The method of Hudspeth *et al.* (1980) was used to recover mtDNA. The pellet was dissolved in 5.0 ml TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 1.0 g of cesium chloride. An additional 4.25 ml of TE and 11.3 g of cesium chloride was required to bring the solution to the proper density and volume for filling the 16 x 76 mm polyallomer ultracentrifuge tubes. The CsCl was dissolved in TE by vigorous inversion and heating. This solution was then added to the dissolved nucleic acids in small aliquots at 30°C. This was done to avoid the formation of precipitate, which occurred frequently and may be due to some remaining DNA-bound proteins. Precipitation of these proteins would reduce the DNA yield. The specific gravity was then checked and adjusted to 1.70 g/ml, which would result in the maximum separation of mitochondrial and nuclear DNA. Since mtDNA has a lower buoyant density than nuclear DNA, it will separate into a band above the nuclear region (Dons *et al.* 1979). This is due to the fact that mtDNA has a lower GC/AT ratio than nuclear DNA, and that AT-rich DNA has a lower buoyant density than DNA with a higher GC content. Addition of bisbenzimidazole, which preferentially intercalates into AT base pairs of the DNA, further reduces the buoyant density of AT-rich DNA sequences by widening the gap between the DNA bases (Hudspeth *et al.* 1980). This aids in increasing the separation

between the nuclear and mtDNA bands in CsCl gradients by a factor of about 2-2.25 (Specht *et al.* 1983). Under ultraviolet (UV) light, bisbenzimidazole also fluoresces, thus making the DNA bands visible.

Addition of 120 μ l of 10 mg/ml bisbenzimidazole to the polyallomer tube was followed by adding the cesium chloride-DNA solution using a Pasteur pipette. The polyallomer tubes were centrifuged in an IEC ultracentrifuge model 60 at 45,000 rpm in a 494 rotor for 48 h at 25°C. Forty-eight hours was the minimum time required for obtaining adequate resolution. An additional 12-24 h resulted in better resolution and tightening of the bands. When viewed under UV light a lower nuclear DNA and an upper AT-rich mtDNA band, about 0.5-1.5 cm apart were observed. MtDNA and nuclear DNA were extracted using either a 1.0 or 5 ml syringe with 20-, 18-, or 16-gauge needles inserted into the polyallomer tube just below the band.

The samples were dialyzed against TE for about 24 h. Purified DNA samples were precipitated with one quarter volume of 7.5 M ammonium acetate and two volumes of cold 95% ethanol. The samples were allowed to precipitate at -20°C, after which they were centrifuged for 10-15 minutes. The liquid was decanted and the pellet washed in 70% ethanol. The ethanol was decanted and the pellet dried in a Speed Vac Concentrator (Savant Instruments Inc., Hicksville, NY) and resuspended in TE.

When it was evident from agarose gel electrophoresis that the mtDNA was still contaminated with nuclear DNA, the samples were redissolved in cesium chloride and the centrifugation repeated as before.

Quantification. The concentration of the sample was determined by measuring the absorbance at 260 nm with a UV-spectrophotometer.

Figure 2: DNA Isolation Techniques

A. MtDNA Isolation: Schematic representation of the steps involved in mtDNA extraction from *Ustilago violacea* (facing page).

- Steps:
1. Cells from a log phase culture were harvested, freeze-dried, extracted with extraction buffer (section 2.3.1) and partitioned against chloroform.
 2. The nucleic acids in the upper phase were precipitated and redissolved in CsCl and centrifuged in a polyallomer tube with bisbenzimidazole.
 3. The mtDNA band was extracted and dialysed.

B. Total DNA Isolation: Schematic diagram of the steps involved in the isolation of total *Ustilago violacea* DNA (page 33).

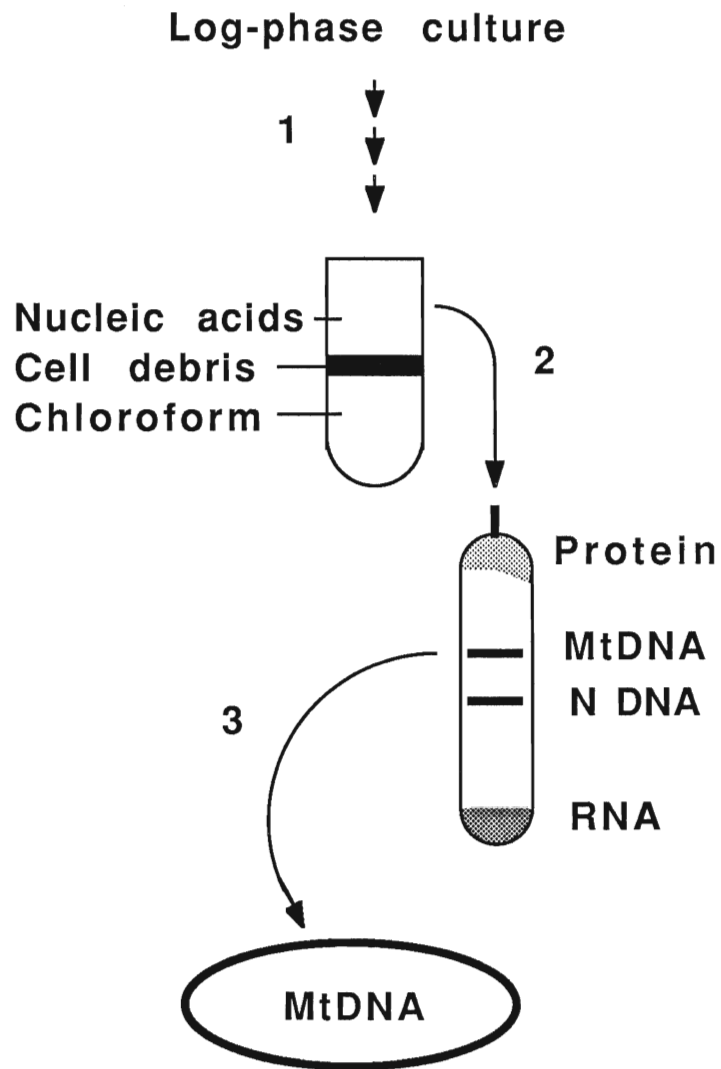
- Steps:
1. (Same as in 'A').
 2. The nucleic acids from the upper phase were precipitated, redissolved in TE and treated with ribonuclease A enzyme followed by chloroform partitioning.
 3. The DNA was precipitated and dissolved in TE.

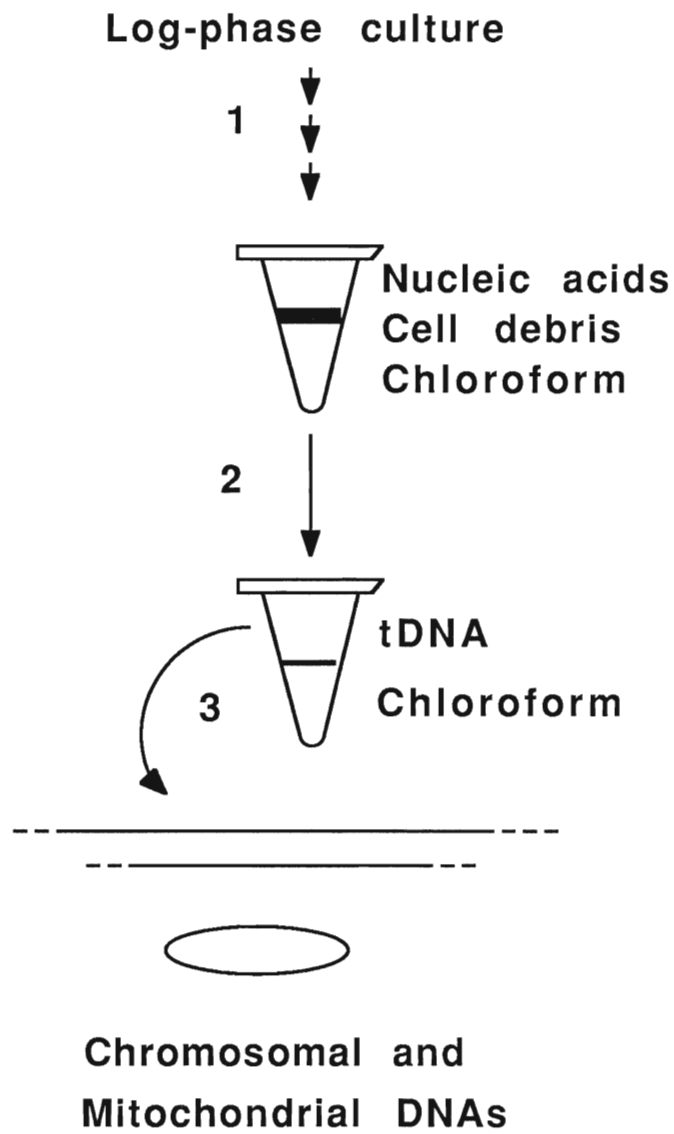
C. Analysis of MtDNA and Total DNA: Schematic representation of the steps involved in the DNA analysis (page 34).

- Steps:
1. DNAs were digested with a restriction enzyme (*EcoR*1).
 2. Resulting DNA fragments were loaded onto an agarose gel and electrophoresed with a sample of DNA size marker.
 3. The gel was stained with ethidium bromide which is able to visualize the DNA bands under UV-light illumination.

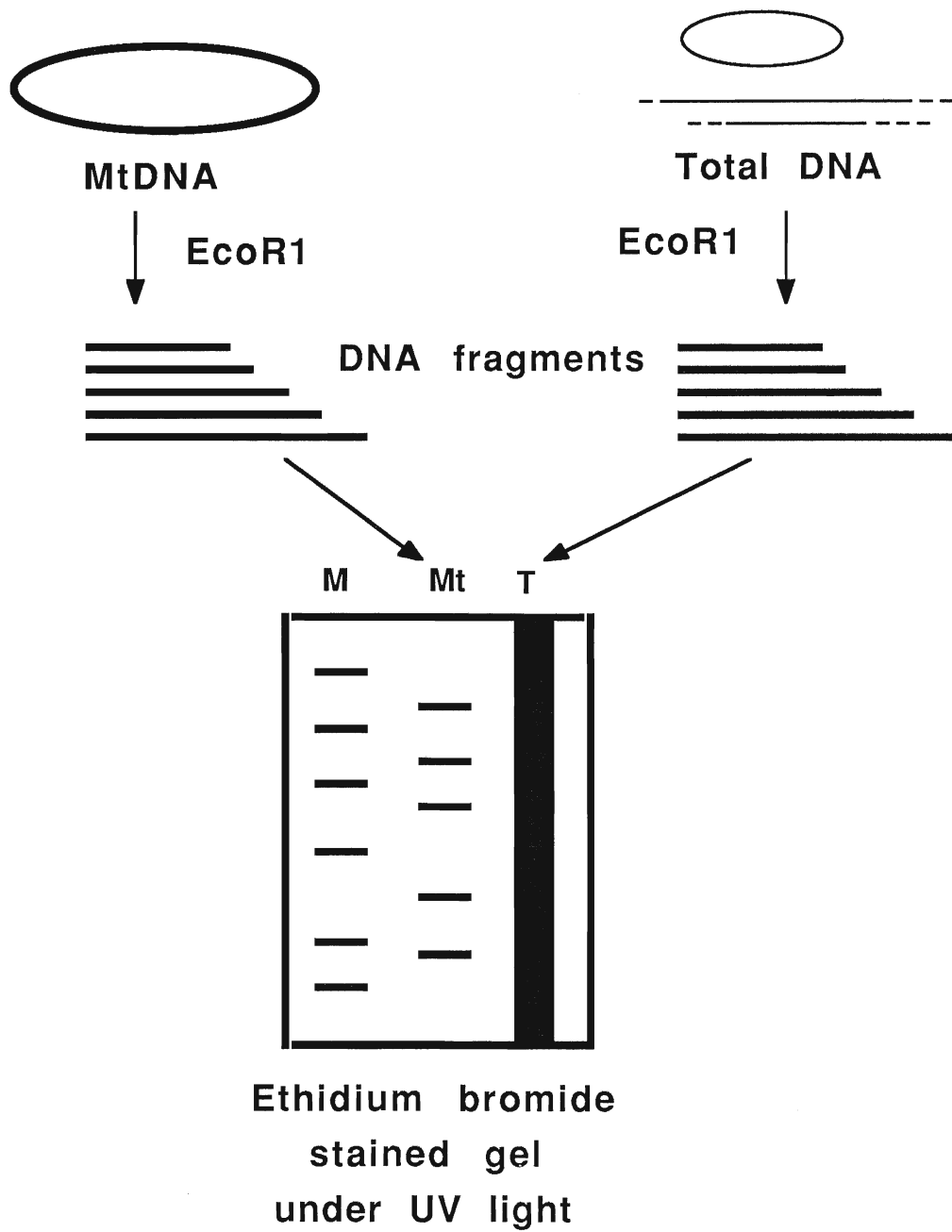
Legend: M - marker lane containing DNA fragments of known sizes
Mt - purified mtDNA sample revealing distinct bands
N - total DNA showing a DNA smear due to overlapping fragments.

A.



B.

C.



One optical density (O. D.) unit corresponds to about 50 mg/ml of DNA. The purity of DNA was also measured by taking the ratio of absorbances at 260 and 280 nm, and of 260 and 230 nm. Pure DNA gives a 260/280 ratio of approximately 1.8 and a 260/230 ratio of 2.0 (Maniatis *et al.* 1982).

2.3.3 Small-Scale DNA Isolation (Fig. 2B)

For the isolation of DNA to be analyzed by Southern blotting and hybridization, the method of Zolan and Pukkila (1986) was used:

To the dried cells, 600 ml DNA extraction buffer (the same as for the mtDNA extraction) was added, and the suspension was mixed by quickly vortexing and allowed to incubate for 15-20 min. at RT. An equal volume of chloroform : isoamyl alcohol (24:1) was added, mixed and allowed to sit for 5 min. before centrifugation in a microcentrifuge for 5 min. The top aqueous layer containing nucleic acids was pipetted into fresh tubes. An equal volume of isopropanol was added to precipitate the nucleic acids. The precipitate was centrifuged for 2 min. and the solution was decanted. The pellet was dissolved in 300 ml TE. 3 ml of RNase A (10 mg/ml) was added and incubated for 30 min. at 37°C.

The chloroform/isoamyl alcohol extraction was repeated. The top aqueous layer of DNA was removed and 75 ml of 7.5 M ammonium acetate and 750 ml cold ethanol added to it to precipitate the nucleic acids. After a 2 min. centrifugation to pellet the DNA, the aqueous phase was discarded. The pellet was dried for 2 min. in a Speed Vac Concentrator, redissolved in 50 ml TE, and stored at 4°C.

2.4 DNA Analysis Techniques (Fig. 2C)

2.4.1 Restriction Endonuclease Digestion

Purified mtDNA was digested with four different restriction endonuclease enzymes, (*EcoRI*, *BamHI*, *HindIII* and *Kpn2*), which all recognize specific 6-base pair DNA sequences and produce fragments by cutting the DNA at these sequences. For a digestion 10 units of enzyme were added to a total volume of 10-20 ml containing the DNA sample, restriction buffer, and distilled water. The digestion was carried out at 37°C for two hours. To stop the reaction the tubes were heated to 65°C for one minute. The *EcoRI* digestion of total mtDNA resulted in the widest range of different fragment sizes with fewest overlapping (similar molecular weight) bands. This enzyme, therefore, was used to digest DNA prior to cloning in plasmid clones.

2.4.2 Agarose-Gel Electrophoresis

At pH near neutrality, DNA is negatively charged and migrates from cathode to anode with a mobility dependent primarily on fragment size and density of the gel. For the DNA fragment sizes expected to be generated by a restriction enzyme recognizing a 6-base pair sequence, a 0.7% agarose gel was prepared (Maniatis *et al.* 1982).

0.7 % Agarose Gel

5 x TBE	25 ml
agarose	0.88 g
distilled water	100 ml

5 x TBE

Tris base	54 g
boric acid	27.5 g
0.5 M EDTA	20 ml
distilled water to	1 liter

The 0.7% agarose gel solution was heated to boiling until the agarose was all dissolved, then cooled to about 50°C and poured into a casting tray with the open ends taped and a well comb in place. After cooling and gelation, the comb and tape were removed and the tray put into the electrophoresis chamber with the wells toward the cathode. 1 x TBE buffer was filled into the chamber until it just covered the gel. Next, 1 ml of bromophenol blue (in 50% glycerol) was added to each DNA sample to be loaded onto the gel. After loading, the samples were separated by electrophoresis at 1.7 V/cm gel length for approximately 16 h.

To visualize the DNA, the gel was placed in 0.5 mg/ml solution of ethidium bromide for 20-30 min. The gel was photographed on a UV transilluminator equipped with a Polaroid camera.

2.4.3 Southern Blotting

A gel of digested total DNA of the strains under study was first immobilized onto a nylon membrane using Southern blotting techniques (Southern 1975). An alkaline transfer technique was employed which was described in the Bio-Rad Bulletin 1233 (K. C. Reed Laboratories, 1414 Harbour Way South, Richmond, CA 94801). This involved acid nicking the nucleic acids by putting the gel in 0.25 N HCl for 10 min followed by a brief rinse in water. The gel was placed on a raised platform with a wick (filter paper) reaching into a tray filled with 0.4 N sodium hydroxide. After covering the gel with 0.4 N NaOH, a nylon DNA-transfer membrane (Biodyne, ICN) cut to the size of the gel was placed on top. The transfer of nucleic acids onto the membrane occurred by passive diffusion, in which the sodium hydroxide solution acted as the transfer agent. A stack of paper towels above the membrane absorbed

the solution through the gel. The membrane was washed in 0.15 M NaCl and 0.015 M sodium citrate, and was air-dried on filter paper for at least 24 h.

2.4.4 Radioactive Labelling of DNA Probes

Radiolabeled probes were obtained by primer extension (Feinberg and Vogelstein 1983). The DNA to be labeled was denatured by boiling and was mixed with a molar excess of oligonucleotide primers. Synthesis was carried out using the Klenow fragment of *E. coli* DNA polymerase I. This enzyme lacks 5'→3' exonuclease activity so that the radioactive product is synthesized exclusively by primer extension rather than by nick translation and is therefore not degraded exonucleolytically. The reaction was carried out at pH 6.6, where the 3' to 5' exonuclease activity of the enzyme is greatly reduced (Lehman and Richardson 1964). These conditions favour random initiation of synthesis, since oligonucleotide primers bound to the template are not degraded enzymatically. The reaction which was employed was as described in the instruction manual provided with the reaction kit by Pharmacia. It involved denaturing about 50 ng of DNA template at 90°C for 15 min in a volume of 31 µl of water. After cooling on ice 10 µl of the Reagent Mix [dATP, dGTP, dTTP, p(dN)6, and buffer] and 2 µl of RNase/DNase-free BSA (bovine serum albumin, V) were added at RT. Next, 5 µl of [α -³²P]dCTP (3000 Ci/mM) and 2 µl of Klenow fragment of DNA polymerase I were added. The reaction was allowed to proceed at RT for 2-24 h. Unincorporated nucleotides were removed by a Sephadex G-50 spun column technique (Maniatis *et al.* 1982). Specific activity was estimated by scintillation counting of TCA precipitated polynucleotides (Berger 1987).

2.4.5 Hybridization of Southern Blot with Labeled DNA

Molecular hybridization is the formation of double-stranded nucleic acid molecules by sequence-specific base pairing of complementary single strands. The protocol followed was by Maniatis *et al.* (1982) and included immersing the Southern blot in 5 ml of a prehybridization solution consisting of 5 x SSC (20 x SSC stock: 3 M NaCl and 0.3 M sodium citrate), 5 x Denhardt's solution (1%, w:v, Ficoll, 1%, w:v, polyvinyl pyrrolidone and 1%, w:v, BSA fraction V), 10%, w:v, dextran sulphate and 1%, w:v, SDS (sodium dodecyl sulfate).

The probe and 120 μ l of 10 mg/ml sheared herring sperm DNA were boiled for 10 min, and were added to the prehybridization solution. The membrane was immersed in this solution and incubated for 16-20 h at 65°C with gentle agitation. Following hybridization, the membrane was washed twice in 250 ml each of the following solutions with agitation: Five min each in 2 x SSC at RT followed by 30 min each at 65°C in 2 x SSC and 1% SDS, which was followed by two 30 min washes at RT in 0.1 x SSC.

Visualization of the labeled regions was done by placing the moist blot onto a 3 MM filter paper soaked in 0.1 x SSC and sealed in a hybridization bag with air bubbles removed. The blot was then exposed to Kodak X-OMAT film in an X-ray cassette with a Cronex intensifying screen for 24 hours at -70°C. The X-ray film was then developed as specified by the manufacturer (Kodak). Subsequent exposure of the blot for a longer or shorter time was done when the intensity of the labelling on the first X-ray was either too low or too high.

The blot was then stored at 4°C until required for another hybridization with a different probe. The blot was first stripped of the

old probe before being reused when it was thought that the probe might interfere with the visualization of the next hybridization. The blot was stripped in a 10-20 ml solution of 2 x SSC and 1% SDS, which was added to the hybridization bag containing the blot. The bag was then placed in a boiling water bath with a piece of styrofoam and a weight covering the bag. After 5-10 min the bag was flipped and boiled for another 5-10 min. When the procedure was finished, the hot solution was immediately removed from the bag and the blot was ready for the next hybridization.

2.5 Cloning of Mitochondrial DNA Fragments (Fig. 3)

Purified mtDNA from isolate UWO-1 was digested with *Eco*R 1 restriction endonuclease and cloned into the plasmid pUC13 which were then transformed into *E. coli* strain JM83 or DH5 α . The pUC plasmids were derived from bacteriophage M13 and are vectors that permit histochemical identification of recombinant clones (Vieira and Messing 1982).

2.5.1 Plasmid Preparation (Fig. 3A)

Plasmid pUC13 was obtained from a culture of *E. coli* strain HB101 carrying the plasmid. The pUC13 plasmid contains genes for ampicillin resistance and a *lacZ'* gene containing a polycloning site. The *lacZ'* gene encodes β -galactosidase, the synthesis of which is induced by isopropylthio- β -D-galactoside (IPTG). Bacteria exposed to IPTG synthesize β -galactosidase and form blue colonies in the presence of chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) (Horwitz *et al.* 1964). A bacterial culture harbouring pUC13 was grown and the plasmid amplified in rich medium according to

Maniatis *et al.* (1982). Plasmid was extracted from the cultures by the boiling lysis method (Holmes and Quigley 1981). The plasmid DNA was purified by centrifugation to equilibrium in cesium chloride/ethidium bromide gradients (Maniatis *et al.* 1982). The purity was checked by agarose-gel electrophoresis.

The pUC13 plasmid has a unique *Eco*R1 recognition site in the *lacZ'* gene. 10 µg of purified plasmid was digested by *Eco*R1 restriction endonuclease. The enzyme was then removed by chloroform/isoamyl alcohol treatment and the DNA precipitated in 95% ethanol. DNA was resuspended in 50 µl of distilled water. To this was added 10 µl of 10 x calf intestinal phosphatase (CIP) buffer, 39 µl of distilled water, and 1 µl (10 units) of CIP enzyme. The reaction was carried out for one hour at 37°C and terminated by heat treating for 15 min at 65°C. DNA was extracted by chloroform partitioning and ethanol precipitation. This procedure removes the terminal phosphate groups exposed on the cut ends of the plasmid, to prevent religation to itself of the plasmid during the ligation with mtDNA fragments.

2.5.2 Ligation Treatment (Fig. 3A)

CIP treated *Eco*R1 pUC13 plasmid was mixed with purified mtDNA which had been digested with *Eco*R1. Samples were heated to 65°C for 15 min. and then cooled to RT. The ligation mixture consisted of ligation buffer, 1 mM dithiothreitol, 1 mM ATP and 1 unit of T4 DNA ligase. The reaction was carried out at 14°C for 24 h.

2.5.3 Transformation of *E. coli* with pUC13 Plasmid (Fig. 3B)

This procedure was adapted from Maniatis *et al.* 1982 and Vieira and Messing 1982. The following solutions were used in this procedure:

Transformation Solution: 100 mM CaCl₂, 250 mM KCl, 5 mM MgCl₂ and 5 mM Tris-HCl, pH 7.5.

LB Medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar (for solid medium) and brought to a volume of 1 litre with water. The pH was adjusted to 7.5 with NaOH.

A single colony of JM83 *E. coli* cells was inoculated into 5 ml of LB liquid medium and allowed to incubate at 37°C with vigorous agitation (200 rpm) for 16 h. A prewarmed (37°C) 500 ml flask with 100 ml LB medium was inoculated with 1 ml of the 5 ml culture and incubated at 37°C at 200 rpm for 2 h. Bacteria were harvested in a 250 ml centrifugation bottle and centrifuged at 3000 rpm for 5 min at 4°C.

Cells were resuspended in 50 ml of cold transformation solution and transferred to an "oakridge" tube. The tube was placed on ice for 10 min. and occasionally mixed. Cells were collected by centrifugation (3000 rpm, 5 min, 4°C) and resuspended in 6.2 ml of the transformation solution. After incubation on ice for 15 min, the cells remained competent for transformation for up to 24 h.

The ligated DNA was allowed to chill on ice and 100 µl of competent JM83 cells was added into each of the tubes. The suspension was gently mixed and allowed to sit on ice for 30 min. The cells were shocked at 42°C for 2 min and allowed to recover for 10 min at RT. Next, 1 ml of LB was added, and the cells were incubated at 37°C for 30 to 60 min.

The cells were then added to test tubes containing 6 µl of 10 mg/ml ampicillin, 4 µl IPTG (200 mg/ml) and 20 µl of X-gal (20 mg/ml, in dimethylformamide). 2 ml of molten soft agar (1%) at 60°C was added and the suspension briefly mixed before pouring onto LB agar

Figure 3: Mitochondrial DNA Probe Construction (section 2.5)

A. Cloning MtDNA Fragments into pUC13 Plasmid (facing page).

- Steps:
1. Purified plasmid pUC13 was digested with *Eco*R1 and treated with calf intestinal phosphatase to remove terminal phosphates.
 2. Purified mtDNA was digested with *Eco*R1
 3. MtDNA restriction fragments were mixed with linearized pUC13 plasmid in the presence of T4 DNA ligase to ligate mtDNA fragments into plasmid.

Legend: Amp R - Ampicillin resistance gene

kb - kilobase pairs

β -gal - β -galactosidase gene

CIP - Calf intestinal phosphatase

*Eco*R1 - Restriction endonuclease enzyme from *E. coli* which cuts specific DNA sequences, indicated by the arrows.

X - any of the DNA fragments generated by *Eco*R1 digestion

B. Transformation of *E. coli* and Analysis of MtDNA Clones (page45).

- Steps:
1. Transformation of *E. coli* with pUC13 clone
 2. *E. coli* were plated onto growth medium containing IPTG, X-gal and ampicillin.
 3. White colonies were picked and grown up for an extraction of their plasmids using the alkaline lysis method.
 4. Plasmids were digested with *Eco*R1, separated on an agarose gel, the gel stained with ethidium bromide and the DNA bands visualized under UV-light.
 5. The gel was blotted (Southern) onto a nylon membrane, which was hybridized with radioactively labeled mtDNA. The blot was then exposed to an X-ray film to visualize the mitochondrial inserts (lanes 1 and 3).

Legend: (same as in Fig. 3A)

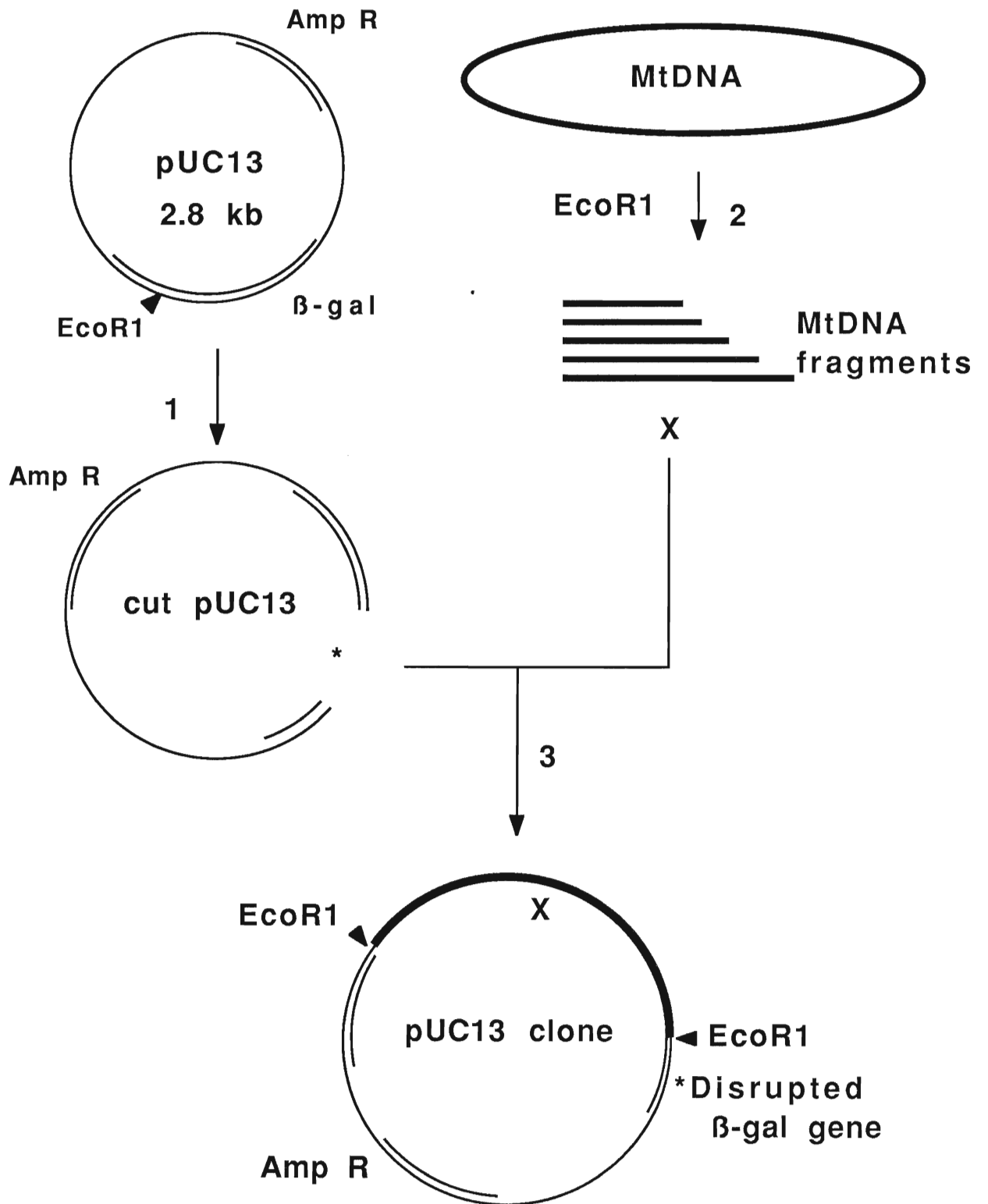
IPTG - (isopropylthio- β -D-galactoside)

X-gal - (5-bromo-4chloro-3-indolyl- β -D-galactoside)

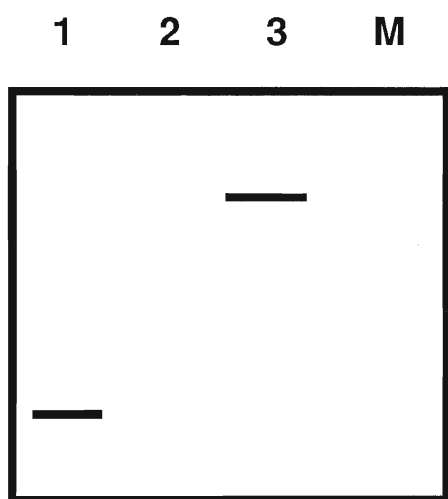
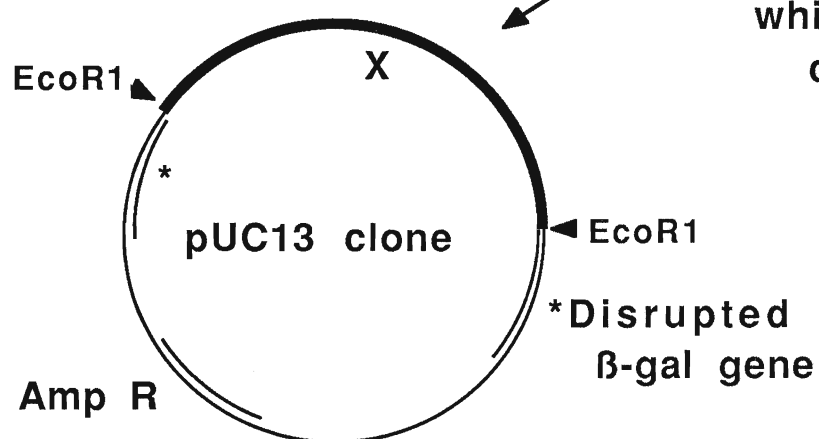
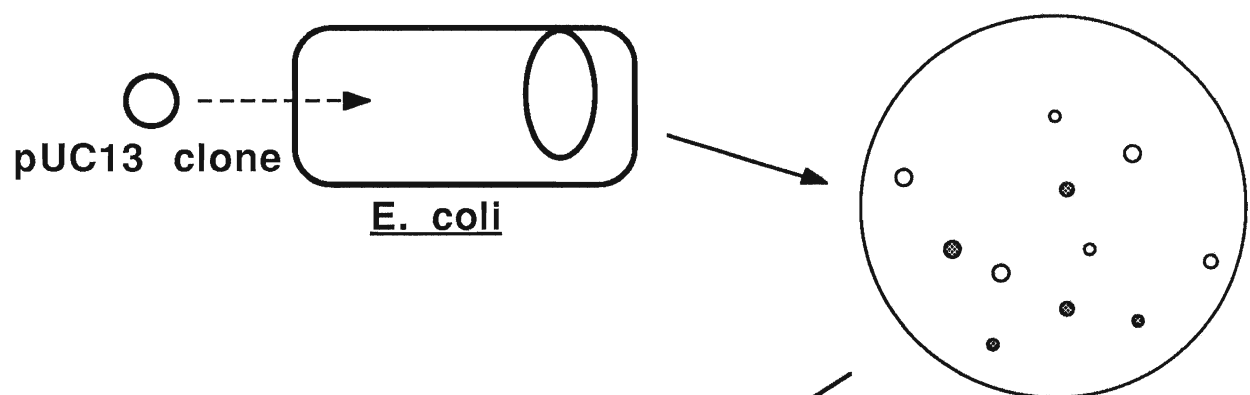
M - DNA size marker

1 to 3 - samples of cut clones, which includes the plasmid and various inserts.

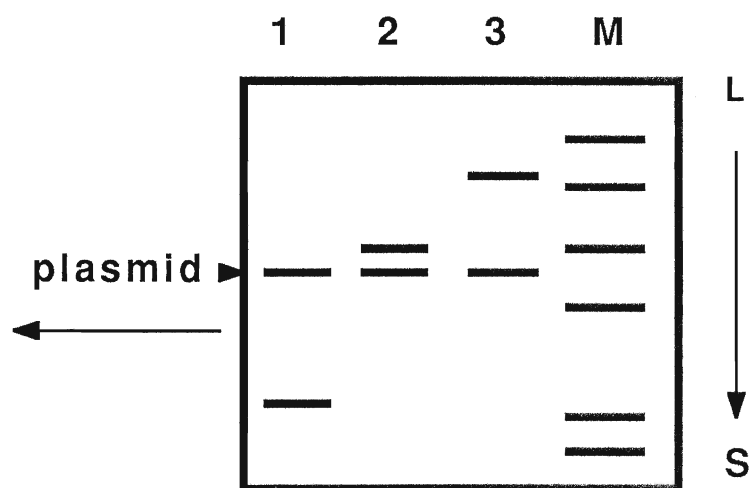
A.



B.



X-ray of blot hybridized
with mtDNA probe



Ethidium bromide stained
gel under UV light

plates containing 50 mg/ml ampicillin. The mixture was evenly distributed on the plate before hardening. After allowing the top-agar to harden for 45 min at RT, the plates were incubated at 37°C for 16 to 24 hours to allow colonies to form. Plates were subsequently stored at 4°C. When colour development was complete the plates were analyzed for putative clones. Since JM83 is unable to grow on LB containing ampicillin, only those cells which had incorporated a pUC13 plasmid were able to grow. If the CIP treatment of the plasmid during its preparation was not 100% efficient, resulting in a portion of the linearized plasmids retaining their terminal phosphate groups, then some of the plasmids would be able to recircularize without incorporating an insert. In this case the *lacZ'* gene might still be intact, and its gene product (β -galactosidase) would be produced. This enzyme metabolizes the chromogenic substrate X-gal. Bacterial colonies harboring a plasmid of this sort would develop a blue color. Since the pUC13 plasmid contains a multiple cloning site within the *lacZ'* gene, insertion of a DNA fragment into the plasmid would result in the disruption of the gene and loss of the the gene product. *E.coli* harboring plasmids with inserts produce white colonies in the presence of X-gal.

2.5.4 Analysis of Clones (Fig. 3B)

White colonies were then analyzed for putative mitochondrial clones. Plasmids containing cloned DNA fragments were extracted from *E. coli* by a modification of the alkaline lysis method described by Birnboim and Doly (1979). White colonies obtained were verified by replating onto LB plates containing a layer of top-agar containing X-gal, IPTG and ampicillin.

The cultures were grown up overnight at 37°C in 5 ml of LB medium including 0.4 mg/ml of ampicillin. An eppendorf tube was filled with the overnight culture of bacterial suspension and centrifuged for one minute. The medium was removed by aspiration. The pellet was resuspended in 10 ml of an ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0 and 4 mg/ml lysozyme.

The mixture was incubated for 5 min at RT, followed by the addition of 200 µl of an ice-cold solution of 0.2 N NaOH, 1% SDS. The contents were gently mixed by inversion of the tube and then allowed to sit on ice for 5 min. 50 ml of an ice-cold solution of potassium acetate (pH 4.8) was made up as follows: To 30 ml of 5 M potassium acetate, 5.75 ml of glacial acetic acid and 14.25 ml of water were added (3 M potassium and 5 M acetate) and stored at 4°C. The tube was inverted and vortexed for 10s and left on ice for 5 min. Following this, the tube was centrifuged for 5 min at 4°C and the supernatant carefully transferred to a fresh tube. An equal volume of phenol/chloroform was added and mixed by vortexing. After a 2 min centrifugation the supernatant was transferred to a fresh tube and 2 volumes of 95% ethanol at RT was added. Contents were mixed and allowed to sit for 2 min before centrifuging for 5 min. The pellet of predominantly plasmid DNA was then washed with 70% ethanol and resuspended in 50 µl of TE. Plasmids were digested with *Eco*R1 and were analyzed by gel electrophoresis.

Inserts had to be verified as mitochondrial in origin, since some nuclear DNA was still present in the purified mtDNA sample. The sizes of the ten identifiable fragments obtained by *Eco*R1 digestion of CsCl purified mtDNA were estimated by comparison to DNA molecular weight standards. The sizes of these fragments were compared to the sizes of

the inserts in the JM83 pUC13 clones. If the the size of the insert was within 10% of one of the 10 mtDNA fragments, it was analyzed further. Nylon membranes having the putative clones immobilized onto them were hybridized with purified mtDNA labeled with [α - 32 P]dCTP. Inserts which showed high levels of radioactively bound probe could be safely assumed to be genuine mitochondrial clones. Clones which showed uncertain results, were radioactively labeled and hybridized to a blot containing both purified mtDNA and purified nuclear DNA. Whether the radioactively labeled probe bound to the mitochondrial or the nuclear DNA of the appropriate size confirmed the identity of the insert.

2.6 Determination of *U. violacea* Isolates Polymorphic in MtDNA

The final step in obtaining mtDNA markers for studying mitochondrial inheritance, involved determining polymorphisms in the sizes of the mitochondrial DNA fragments obtained by digestion of DNAs from various *U. violacea* isolates with a restriction enzyme, in this case, *Eco*R1. Different isolates were examined. Total genomic DNAs were digested, fractioned according to size by agarose gel electrophoresis, and blotted onto a nylon membrane. The nylon membrane was then hybridized with one or more radioactively labeled mitochondrial clones. Polymorphisms were evident when clones hybridized to different molecular weight regions in particular *U. violacea* isolates.

2.7 Crosses

The three isolates were crossed reciprocally on water agar and then transferred to complete medium where one drop of 10^{-5} M α -tocopherol (vitamin E) was added. Mated cells form hyphae which continue to elongate until the vitamin E is exhausted or oxidized.

Following this, the cultures revert to haploid sporidial growth forming colonies that are bisected for the nuclear parental type. Bisected colonies were readily detected in crosses involving a yellow-colored parent and a pink (wild type) parent. Bisected colonies were streaked to obtain single yellow and pink colonies which were also analyzed for other nuclear markers.

DNAs from progeny of the crosses and from the parental types (original, uncrossed) were digested with *Eco*R1 and separated by agarose gel electrophoresis. Separated DNA smears were fixed onto a nylon membrane by Southern blotting. The Southern was hybridized with a radioactively labeled mitochondrial clone known to be polymorphic among the parental strains of the cross. Labeled blots were then exposed to X-ray film and the resulting fragment patterns compared with those of their parents.

RESULTS

3.1 Estimate of Mitochondrial Genome Size

Isopycnic gradient centrifugation of total *U. violacea* DNA in cesium chloride solution at a density of 1.68-1.71 g/ml with bisbenzimidazole resulted in two bands forming near the center of the tube, usually 0.5-1.5 cm apart. The upper band is enriched for mtDNA (Dons *et al.* 1979), since mtDNA has a molar mass slightly less than that of nuclear DNA because of its relatively high AT content (approximately 70% for most fungi).

The problem of contaminating nuclear DNA was frequently encountered. Most of the difficulty had to do with the efficiency with which the cesium chloride/bisbenzimidazole gradient centrifugation was able to separate the mtDNA from the nuclear, and the accuracy with which the top mitochondrial band could be extracted. Separation of the DNAs depended greatly on maintaining the density near 1.70 g/ml. Centrifuging at 45,000 rpm for 48 hours was the minimum time required for adequate resolution. An additional 12 to 24 hours resulted in better resolution and tightening of the bands, facilitating extraction of a purer and more concentrated sample. Occasionally a third, intermediate band was observed, which fluoresced with about the same intensity as the mtDNA band above it. This phenomenon was observed especially when the cells were ground prior to the DNA extraction. This band may be enriched for ribosomal DNA sequences which are higher in their AT-content than other nuclear DNA sequences. This third band has also been observed by Garber and Yoder (1983), Specht *et al.* (1983) and Klassen *et al.* (1987). In each of these cases the ability to resolve the third intermediate band was also found to vary with the preparation and it was confirmed that this band is highly enriched for nuclear rDNA.

Purified mtDNA was cut into fragments by digestion with restriction endonuclease enzymes *HindIII*, *EcoR1*, *BamH1* and *Kpn2*. Agarose gel electrophoresis showed that the *EcoR1* digest resulted in the largest range of fragments with the fewest overlapping bands (Fig. 4, lane 2). This is a useful feature for RFLP analysis and *EcoR1* was subsequently used in DNA cloning trials and RFLP analysis. Ten fragments were identified in a digestion of UWO-1 mtDNA with *EcoR1*. Their estimated sizes are given in Table 2. From these estimates the total mitochondrial genome size of UWO-1 was calculated to be 45 ± 4 kb. The sizes were calculated from several gels with the variation as indicated. Fragment sizes for which no variation is indicated were derived from analysis of a single gel. It was difficult to obtain an accurate measurement for the large fragments since they do not separate very well and travel only a short distance in the gel. In addition, for gels made up with TBE, skewing of high molecular weight DNA fragments frequently occurred resulting in uneven band migration and dubious size estimates. This problem was rectified by use of TAE buffer instead.

DNA samples obtained from a number of isolates often did not digest well with *EcoR1* restriction endonuclease making RFLP analysis difficult. The most probable explanation for the poor digestion efficiency may be that the DNA contained impurities that reduced enzyme efficiency. Alteration of culture growth conditions or DNA extraction methods did not result in any consistently improved digestion efficiency. These attempts involved 1) using log phase cultures, 2) reducing the time used for DNA precipitation in order to reduce co-precipitation of contaminants, 3) using various amounts of cells, 4) treating extracts with

Figure 4: Agarose-Gel Electrophoresis of Mitochondrial and Nuclear DNA digested with Three Different Restriction Enzymes Stained with Ethidium Bromide.

Legend: Lane 0: DNA size marker; sizes of the fragments are indicated in kilobases
Lane 1: *Hind*III digest of mtDNA
Lane 2: *Eco*R1 digest of mtDNA
Lane 3: *Kpn*2 digest of mtDNA
Lane 4: *Hind*III digest of nuclear DNA
Lane 5: *Eco*R1 digest of nuclear DNA
Lane 6: *Kpn*2 digest of nuclear DNA

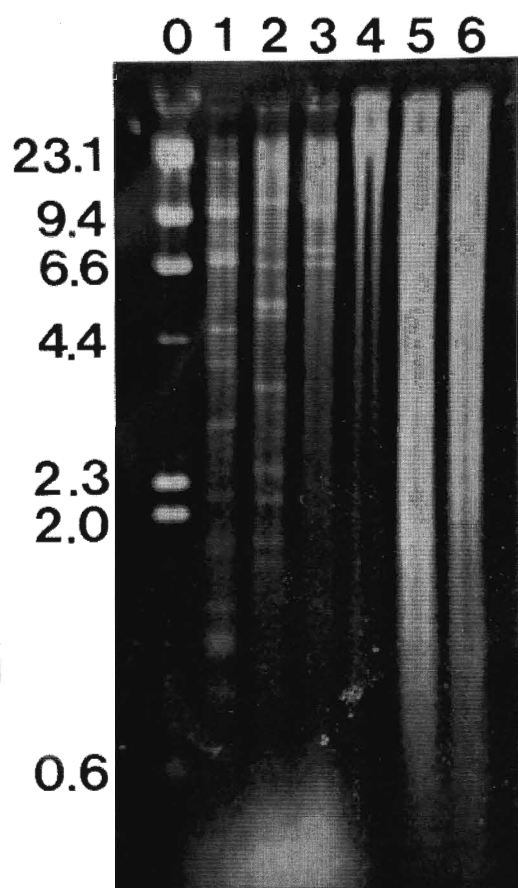


Table 2. UWO-1 Mitochondrial DNA Fragments Generated with *Eco*R1 Digestion, and the Corresponding Clones that were Obtained.

Mitochondrial Fragment	Estimated Size (kilobases)	Plasmid Clone	Number Obtained
F1	11.0 \pm 1.0 kb	-	0
F2	6.8 \pm 0.4 kb	pUvm1.2	3
F3	6.0 \pm 0.7 kb	pUvm1.3	1
F4	5.3 \pm 0.3 kb	pUvm1.4	3
F5	4.8 kb	pUvm1.5	2
F6	3.5 \pm 0.2 kb	pUvm1.6	5
F7	2.4 kb	pUvm1.7	1
F8	2.2 \pm 0.2 kb	pUvm1.8	1
F9	1.8 kb	pUvm1.9	1
F10	1.6 kb	pUvm1.10	1
Combined	45 \pm 4 kb	-	-

proteinase K and 5) washing the resulting DNA pellet with 95% and 70% ethanol followed by drying *in vacuo*.

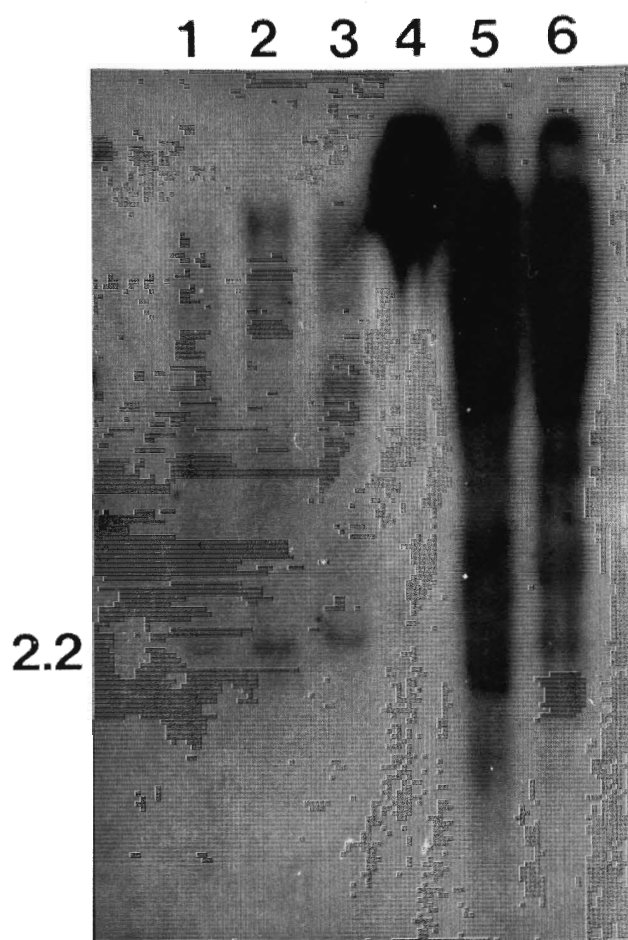
3.2 Mitochondrial Probe Construction

Cesium chloride purified mtDNA was digested with *Eco*R1 and 20 to 40 ng of mtDNA fragments was mixed with 15 ng of prepared plasmid in a ligation solution. Following ligation the solution was added to competent *E. coli* JM83 cells which were prepared experimentally or DH5 α cells which were commercially obtained. Following transformation the cells were plated onto growth medium containing ampicillin and X-gal. Transformation of JM83 yielded 47 colonies and DH5 α 72 colonies. Eight additional clones were obtained from earlier cloning experiments. Of these 127 clones, 104 were screened further: plasmid DNA was isolated from each of these clones, digested with *Eco*R1, and separated on agarose gels. 77 clones contained plasmids with inserts. 18 of these clones were subsequently confirmed by hybridization with radioactively labeled mtDNAs to contain mtDNA inserts (Table 2). Except for the largest mitochondrial fragment (F1), it appears that at least one clone was obtained for each mitochondrial fragment identified in the *Eco*R1 digest of strain UWO-1. Several clones were found to contain nuclear DNA. One of these nuclear DNA clones (about 10 kbp) hybridized to several fragments of nuclear DNA and weakly to mtDNA fragment F8 (2.2 kbp)(Fig. 5). This clone presumably contains all or part of the nuclear ribosomal RNA gene repeat.

Figure 5: Hybridization of a Radioactively Labeled Nuclear Clone Containing Putative rDNA Sequences to a Blot of Nuclear and MtDNAs Digested with Three Restriction Enzymes.

Legend: Lane 1: *HindIII* digest of mtDNA
Lane 2: *EcoR1* digest of mtDNA
Lane 3: *Kpn2* digest of mtDNA
Lane 4: *HindIII* digest of nuclear DNA
Lane 5: *EcoR1* digest of nuclear DNA
Lane 6: *Kpn2* digest of nuclear DNA

The size marker is given in kilobases



3.3 RFLP Analysis

3.3.1 Mitochondrial Markers

Cultures of isolates listed in Table 1 were germinated, grown and total DNA extracted from them. MtDNA *Eco*R1 restriction patterns of nine isolates were analysed for RFLPs with as many as five different plasmid clones. Fragment sizes recognized by the radioactively labeled mitochondrial clones among isolates of *U. violacea* are given in Table 3. In general, fragments F3, F6 and F8 were the same size in all isolates. Fragments F2 and F4 (clones pUvm1.2 and pUvm1.4) revealed a significant and easily recognizable polymorphism between isolates collected in the U. K. and those of Berlin and Hungary. In the U. K. isolates (UWO-1, 3, 25 and 27) the F2 mitochondrial fragment was 7.2 ± 0.5 kbp, while in isolates BU-1, 3, 4, 5 and 6 recognized a fragment measuring 10.3 ± 0.7 kbp. Similarly, the F4 fragment in UWO isolates was about 5 kbp, while in BU isolates it was about 7 kbp (Table 3). These RFLPs were used as markers in crosses involving isolates UWO-1 and BU-1 and BU-3.

3.3.2 Crosses

Reciprocal crosses were performed between isolates UWO-1 and BU-1, UWO-1 and BU-3 and BU-1 and BU-3. Initial results suggested that there was a small size difference in the F2 fragment between isolates BU-1 and BU-3. This, however, was negated in subsequent experiments and no RFLP information, necessary for determining mitochondrial inheritance, could be gained from this cross. Suspensions of mated cells in 10^{-5} M α -tocopherol were plated dilutely onto complete medium (CM), where they were observed to form hyphae. A high percentage of mating was obtained in all of the crosses and

Table 3. Restriction Fragment Sizes of *Eco*R1 digested MtDNAs from Different *Ustilago violacea* Isolates.

Clone	UWO-1	UWO-3	UWO-25	UWO-27	BU-1	BU-3	BU-4	BU-5	BU-6
pUv m1.2	7.0 ± 0.5	7.0 ± 0.5	7.0 ± 0.5	7.4 ± 0.2	10.3 ± 0.7	10.3 ± 0.7	10.3 ± 0.7	10.3 ± 0.7	10.3 ± 0.7
pUv m1.3	5.5	N. A.	N. A.	6.8 ± 0.3	6.8	6.8	N. A.	N. A.	6.8 ± 0.3
pUv m1.4	5.3 ± 0.3	5.2 ± 0.3	5.1+/-0.4	4.8	7.5 ± 0.5	7.2	6.8	N. A.	7.3 ± 0.3
pUv m1.6	3.4 ± 0.2	3.4 ± 0.1	3.4 ± 0.1	3.15	3.5 ± 0.1	3.3	3.15	N. A.	3.4 ± 0.3
pUv m1.8	2.2 ± 0.1	2.25	2.3 ± 0.2	2.15	2.4 ± 0.2	N. A.	N. A.	N. A.	N. A.

N. A.: These fragments were not analysed

virtually all the mated cells formed hyphae. The hyphae ranged in size from 2-10 times the length of a cell. Thus, the cytoplasm from both cells appeared to be transferred in entirety to the hypha.

Following the depletion of α -tocopherol, the mated hyphal dikaryon reverted to haploid sporidial growth on CM. Since the strains used in crosses had different color phenotypes, colonies of cells originating from a mated pair resulted in a colony bisected in color. A single colony from each half of, on average, nine bisected colonies per cross was grown up and analyzed for nuclear markers and the two polymorphic mtDNA fragments F2 and F4. Table 4 shows the results of the mitochondrial types recovered from the various progeny of the crosses. In most of the progeny from these crosses, either one or the other parental mitochondrial RFLP was detected. A strong bias towards the inheritance of mitochondria from the a_2 -nuclear type parent is evident from the results. Overall, the a_2 -nuclear type mitochondrion was transmitted in 56 out of 73 progeny analyzed, or in 77% of the cases.

In two instances, both parental mitochondrial types were detected in the progeny, but in varying concentrations. In the cross between BU-1 a_1 and UWO-2.C2u5, two progeny with the a_2 nuclear type contained both parental mtDNA markers (Fig. 6). DNA analyzed in lane 2 showed an intense signal for the fragment characteristic of mtDNA from the a_2 parent and a much weaker signal for the fragment of the a_1 parent. Had the X-ray film not been overexposed, the weaker band in lane 2 might not have been detected. In the other instance the reverse situation with respect to the intensities of the two bands was observed. A similar result was obtained with the other mtDNA probe (Table 4). DNAs analyzed had been extracted from colonies that were derived from

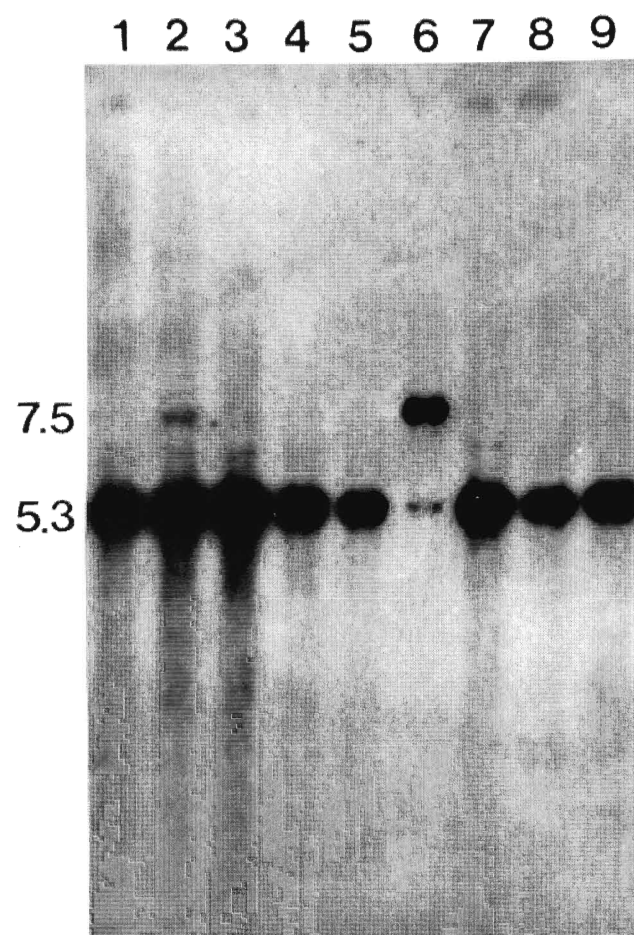
Table 4. The Mitochondrial Types, According to Parent-Type, of Progeny from Crosses Between Isolates of *U. violacea*.

Cross	Progeny Nucl. Genotype	N	Plasmid Probe	<u>Origin of Mitochondria:</u>	
				a ₁ Parent	a ₂ Parent
UWO-1.C2 X BU-1a2	a ₁ , y, his-	11	pUvm1.2	1	10
			pUvm1.4	1	10
	a ₂ , +	9	pUvm1.2	1	8
			pUvm1.4	1	8
BU-1a1 X UWO-2.C2u5	a ₁ , +	8	pUvm1.2	5	3
			pUvm1.4	5	3
	a ₂ , y, his-, uvs	10	pUvm1.2	1 m1>m2	8, 1 m2>>m1
			pUvm1.4	1 m1>m2	9
UWO-1.C2 X BU-3a2	a ₁ , y, his-	10	pUvm1.2	5	5
			pUvm1.4	5	5
	a ₂ , +	10	pUvm1.2	0	10
BU-3a1 X UWO-2.C2u5	a ₁ , +	9	pUvm1.2	4	5
			pUvm1.4	4	5
	a ₂ , y, his-, uvs	6	pUvm1.2	0	6
			pUvm1.4	0	6
Combined	a ₁	38	pUvm1.2/4	15 (40%)	23 (60%)
	a ₂	35	pUvm1.2/4	2 (6%)	33 (94%)

m1 - mitochondria from a₁ parent; **m2** - mitochondria from a₂ parent; **y**-yellow colour; **his**⁻ - histidine requiring; **uvs** - UV-light sensitive; **+** - wild type.

Figure 6: The Presence of Both Parental Mitochondrial Types in DNA Samples Derived from Single Cells.

DNAs from ten progeny with the α_2 -parental nuclear type from a cross involving BU-1a₁ and UWO-2.C2u5 were digested with *Eco*R1, separated by agarose gel electrophoresis, Southern blotted and hybridized with plasmid pUvm1.2. Lanes 2 and 6 show detectable amounts of both parental mtDNA types recognized by the probe. Sizes of the two fragments are given in kilobases.



single cells by streak purification of a bisected colony. The recovery of both mitochondrial types in a DNA sample derived from a colony of this type from a bisected colony resulting from a cross, was strong evidence that mitochondrial inheritance was biparental.

No recombinants were detected in this analysis. In the event of recombination between the two parental mitochondrial types encompassing one of the RFLP markers, one would expect a recombinant mtDNA molecule including one RFLP marker from the a_1 parent and the other from the a_2 parent. The inability to recover recombinants in this study did not rule out that recombination occurred, since recombinant molecules must be able to establish themselves in a large pool of other molecules. In most instances one would expect such recombinants to be lost, mainly due to genetic drift (see section 1.4.3).

In addition to the evidence for biparental inheritance, the analysis of nuclear and mitochondrial genotypes from bisected colonies (Table 5) demonstrated that mitochondria are transferred between mated cells. Since the hypha formed from only one of the cells in a mated pair, evidence to support mitochondrial transfer through the conjugation tube would be the recovery of both mitochondrial types from a bisected colony. Columns 1 and 4 of Table 5 provide that evidence.

3.4 Analysis of Results

Several hypotheses were proposed to further examine the results and to determine the factors which might affect mitochondrial transmission among these isolates of *U. violacea*. In each case a single-tailed X^2 test was performed at the 95% confidence level. With one degree of freedom, the critical X^2 value is 3.84. A value of $P(X^2)$ of less than 0.05 would indicate that the observed frequencies are significantly

Table 5. Analysis of Nuclear and Mitochondrial Genotypes from Bisected Colonies

Cross	Genotypes Recovered from Bisected Colonies:			
	a_1m1/a_2m2	a_1m2/a_2m2	a_1m1/a_2m1	a_1m2/a_2m1
UWO-1.C2 X BU-1a ₂	1	7	0	1
BU-1a ₁ X UWO-2.C2u5	4	3	1	0
UWO-1.C2 X BU-3a ₂	5	5	0	0
BU-3a ₁ X UWO-2.C2u5	2	4	0	0
Totals	12	19	1	1

m1 - mitochondrial genotype of a_1 parent; **m2** - mitochondrial genotype of a_2 parent

different from the frequencies predicted by the hypothesis (Sokal and Rohlf, 1981).

3.4.1 Isolate Differences

Mitochondria from one of the isolates may have been inherited preferentially over mitochondria from the other isolates. The following two hypotheses were proposed to test this possibility.

Hypothesis 1: In reciprocal crosses of UWO-1 with BU-1, mitochondria from these parents were inherited equally well in the progeny.

Hypothesis 2: In reciprocal crosses of UWO-1 with BU-3, mitochondria from these parents were inherited equally well in the progeny.

The results are given in Table 6. Both analyses yielded a $P_{(X^2)}$ greater than 0.1. Therefore, there is insufficient evidence to reject hypotheses 1 and 2 at the 95% confidence level. Mitochondria of the three isolates studied are inherited equally well in the progeny of the crosses performed.

3.4.2 Mating Type Differences

It was possible that mating type, which is known to influence the formation of the conjugation tube (Poon and Day 1974) affected mitochondrial inheritance. Therefore the following hypotheses were proposed to test any relationship between the mating type and mitochondrial type of the progeny (Table 7):

Hypothesis 3: Mitochondria from both a_1 and a_2 parents of reciprocal crosses of UWO-1 with BU-1 and BU-3 were inherited equally well in the a_1 progeny.

Table 6. Recovery of Progeny with the Same Mitochondrial Genotype as the Parent Isolate.

Cross	Mitochondrial Origin			Expected	$\chi^2_{(.05)}$	$P(\chi^2)$
	UWO-1	BU-1	BU-3			
UWO-1 X BU-1	14	24	-	19	2.63	$P>0.1$
UWO-1 X BU-3	16	-	19	17.5	0.257	$P>0.1$

Table 7. Parental Origin of Mitochondria in a₁ Progeny, a₂ Progeny and Combined Results in Crosses of UWO-1 with BU-1 and BU-3.

Progeny	<u>Origin of Mitochondria:</u>		Expected	$X^2_{(.05)}$	P(X^2)
	a ₁ Parent	a ₂ Parent			
a ₁	15	23	19	1.68	P>0.1
a ₂	2	33	17.5	27.5	P<0.001
Combined	17	56	36.5	20.8	P<0.001

Hypothesis 4: Mitochondria from both a_1 and a_2 parents of reciprocal crosses of UWO-1 with BU-1 and BU-3 were inherited equally well in the a_2 progeny.

Hypothesis 5: Mitochondria of a_1 parents were inherited equally well as those of a_2 parents in crosses of UWO-1 with BU-1 and BU-3.

While there was insufficient evidence to reject hypothesis 3 ($P>0.1$), hypothesis 4 and 5 are clearly rejected ($P<0.001$): Mitochondria of a_2 parents were preferentially inherited in a_2 progeny. Progeny with a_1 mating type received mitochondria from either parent equally well. Therefore there appeared to be a significant asymmetry in mitochondrial inheritance that appeared to be related to mating type.

3.4.3 Transfer of Mitochondria through the Conjugation Tube

The results in tables 4 and 5 indicated clearly that mitochondrial inheritance in *Ustilago violacea* was biparental. The observation that two of the progeny contained both mitochondrial types (Fig. 6) and that 40% of the bisected colonies (for nuclear genotype) were also bisected for mitochondrial type indicated that this mode of inheritance must involve transfer of mitochondria through the conjugation tube. The question whether mitochondria were transmitted through the conjugation tube in both directions remained unresolved. The ability to distinguish the two mating type cells under the microscope in order to tell from which mating type cell of the mated dikaryon the hypha was developing would provide a method for addressing this question. Progeny from hyphae which originate from cells of known mating type could be examined for mitochondrial and nuclear genotypes. Several attempts to mark cells by staining were unsuccessful. Cell cultures stained with Crystal violet or Saffranin did not mate. The lipid stain

Sudan III was also tried, but failed to penetrate the cells, leaving them unstained. Cells stained with ethidium bromide were only transiently labeled, with very few cells retaining the stain. Those that retained the stain appeared very bright under the UV-light microscope, but they did not mate.

Due to a lack of a suitable stain to mark the cells, another method to distinguish the mating types was attempted with mixed success. Cells from actively growing cultures were mated with cells obtained from cold storage (4-8 weeks old). Log-phase cultures consisted mainly of narrow, elongated, oval-shaped cells while the stationary-phase cultures were characterized by thick dark cells containing large vacuoles. Reciprocal matings of young and old cultures of opposite mating type for isolates UWO-1, BU-1 and BU-3 were attempted. It was found that crosses involving UWO-1 could not be reciprocally mated, since old UWO-1 cultures did not exhibit the characteristic "old" morphology in a uniform manner and could not be accurately distinguished from young cultures. Therefore only young cultures of UWO-1 were employed in crosses. BU-1 and BU-3, however, were reciprocally crossed. The analysis was often difficult, because the old and young cells of a mated dikaryon could, in the majority of cases, not be determined. Added to this, the mating frequency was often very low since most of the cells in the stationary phase cultures were inviable. The results are shown in Table 8. Due to the paucity of counts obtained and a degree of uncertainty about the identity of the cells, the frequency estimates should be regarded with caution. Crosses involving those strains analyzed for inheritance appear to indicate roughly equal amounts of hyphal formation from both mating types. However, a strong bias in the age was also apparent (57% of hyphae formed from young cells) and the significance of this factor could

not be determined since appropriate reciprocal crosses could not be performed for reasons stated earlier. Instead, other crosses were performed with isolates BU-1 and BU-3 that could be analyzed in reciprocal crosses. These results confirmed that age does have a minor effect on the origin of hyphal formation, favoring the young cells in 55% of the cases. They also indicated a strong bias of 71% for the formation of hyphae on a_2 cells, unlike the results involving crosses with UWO-1 for which the difference was insignificant ($P>0.1$). In spite of the uncertainty, one fact was apparent: that hyphae were able to form from either of the mated cells.

Since it was impossible under present circumstances to directly test for the directionality of mitochondrial transfer through the conjugation tube, it would be possible to do so indirectly by proposing a few models and comparing the expected inheritance results against those actually obtained.

Model 1 assumes bidirectional transfer of mitochondria (Fig. 7A). If bidirectional transfer is the case, one would expect that the four possible nuclear/mitochondrial genotypic outcomes of the progeny would occur in a 1 : 1 : 1 : 1 ratio.

Model 2 assumes that mitochondrial transfer occurs unidirectionally. In this case mitochondria are transferred from the a_2 into the a_1 parent cell, but not from the a_1 into the a_2 parent cell (Fig. 7B). In this situation one would expect the four possible nuclear/mitochondrial genotypic outcomes for the progeny, a_1m_1 , a_2m_2 , a_1m_2 , a_2m_1 , to occur at a 1 : 3 : 3 : 1 ratio, respectively.

The models were tested for their ability to explain the mitochondrial inheritance results, and the results are shown in Tables 9 and 10.

Table 8. Analysis of Hyphal Formation from Various Crosses Involving Young and Old Cultures.

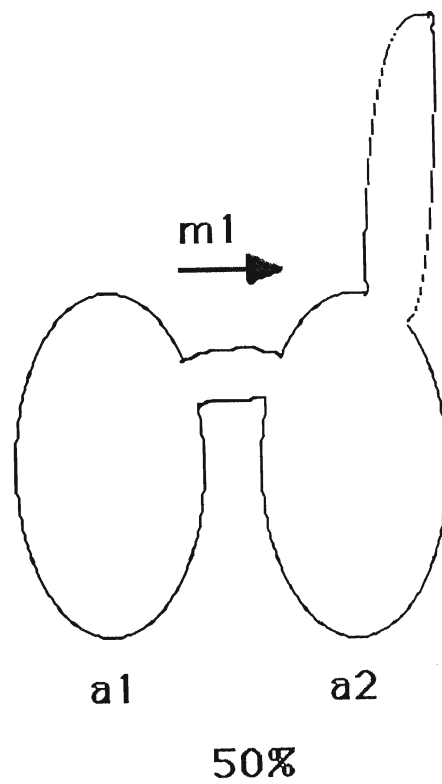
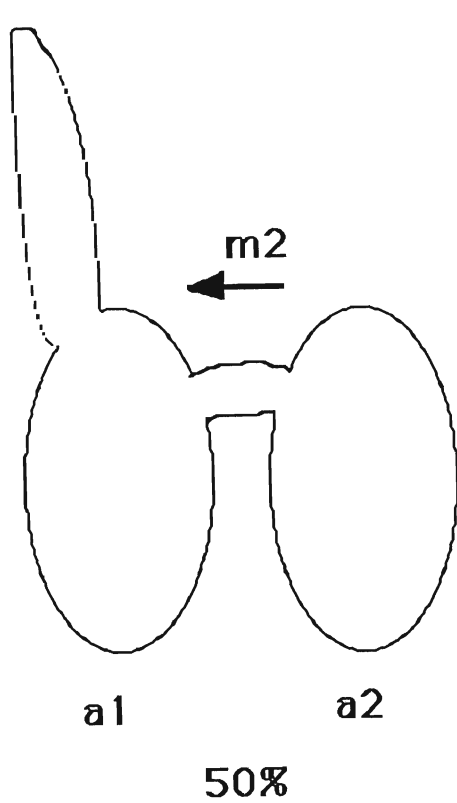
Crosses		Y : O	% Young	#a ₁	#a ₂
Young	Old				
UWO-1.C2	BU-1a ₂	16 : 14	53	16	14
UWO-2.C2u5	BU-1a ₁	72 : 65	53	65	72
UWO-1.C2	BU-3a ₂	56 : 24	70	56	24
UWO-2.C2u5	BU-3a ₁	43 : 37	54	37	43
BU-1Ca ₁	BU-3a ₂	50 : 68	42	50	68
BU-1Ca ₂	BU-3a ₁	75 : 47	61	47	75
BU-3a ₁	BU-1Ca ₂	14 : 50	22	14	50
BU-3a ₂	BU-1Ca ₁	85 : 15	85	15	85
Combined		411 : 320	56	300(41%)	431(59%)

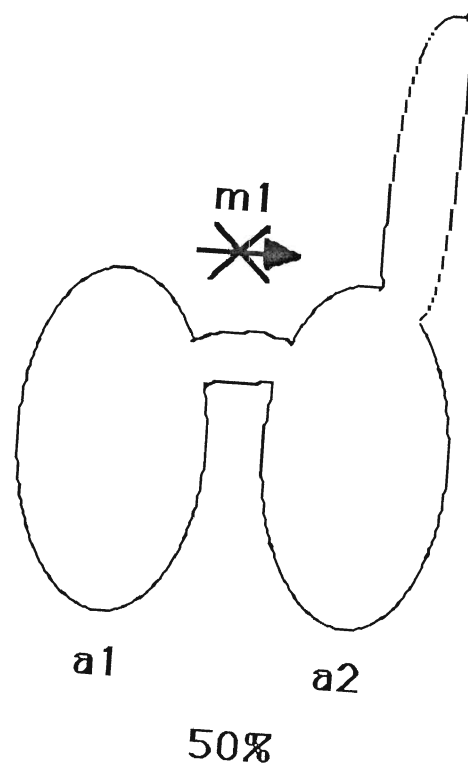
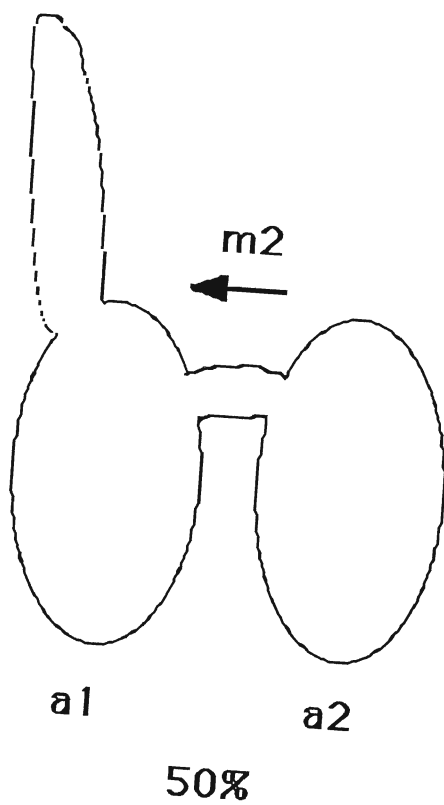
Figure 7: Models of Mitochondrial Inheritance:

A. Bidirectional Transfer: When mitochondria are transmitted bidirectionally, one would expect a 1 : 1 : 1 : 1 ratio of the four possible nuclear/mitochondrial outcomes.

B. Unidirectional Transfer: When mitochondria are transmitted unidirectionally, from the a_2 to the a_1 cell, and if hyphae form equally well from both mated cells, then one would expect a 1 : 3 : 3 : 1 ratio of a_1m_1 : a_1m_2 : a_2m_2 : a_2m_1 progeny.

Legend: a_1 - nuclear type of a_1 parent
 a_2 - nuclear type of a_2 parent
 m_1 - mitochondrial type of a_1 parent
 m_2 - mitochondrial type of a_2 parent

A

B

Model 1, which states that all of the four possible nuclear/mitochondrial combinations occur at equal frequency was clearly rejected ($P < 0.001$): The number of progeny with the a_2m1 combination occurred too infrequently and the a_2m2 combination occurred too frequently to fit the model.

Model 2 states that the four possible progeny occur in a 1 : 3 : 3 : 1 ratio. This model was also rejected at the 95% confidence level ($P < 0.05$) due to the small number of progeny obtained with the a_2m1 combination. However, the results are significant at the 99% confidence level ($0.02 > P > 0.01$).

Table 9. Bidirectional Model: Comparison with the Results Obtained.

	a_1m_1	a_2m_2	a_1m_2	a_2m_1	X^2 Total	$P(X^2)$
Expected	18.25	18.25	18.25	18.25	-	
Actual	15	33	23	2	-	
X^2	0.59	11.92	1.24	14.47	28.22	$P<0.001$

Table 10. Unidirectional Model: Comparison with the Results Obtained.

	a_1m_1	a_2m_2	a_1m_2	a_2m_1	X^2 Total	$P(X^2)$
Expected	9.125	27.375	27.375	9.125	-	-
Actual	15	33	23	2	-	-
X^2	3.78	1.16	0.70	5.56	11.20	0.02>P>0.01

DISCUSSION

Isopycnic gradient centrifugation of *U. violacea* nucleic acids normally resulted in the resolution of two DNA bands. The upper band was enriched for mtDNA and the other for nuclear DNA. The DNAs were not as widely separated as the corresponding DNAs reported by Specht *et al.* (1983) working with *Schizophyllum commune*. They stated that the two bands had buoyant densities of 1.684 (mtDNA) and 1.718 (nuclear DNA). It is therefore likely that the difference between nuclear and mtDNA buoyant densities in *U. violacea* is considerably less. This was the case with *Ustilago cynodontis*, where the mtDNA had a buoyant density of 1.698 and the nuclear DNA 1.713 (Mery-Drugeon *et al.* 1981). A closely related species, *U. maydis*, has a very similar buoyant density (1.697) (Banks 1973). It is probable that *U. violacea* DNA has buoyant densities that are similar to the two *Ustilago* species above, which may explain why the separation of the two bands was not as wide as reported by Specht *et al.* (1983). A large variation in buoyant densities of mtDNAs is evident between fungi. In *Agaricus brunnescens*, the nuclear DNA is 1.6958 g/ml and the mtDNA is 1.6799 g/ml (Arthur *et al.* 1982). In the Chitridiomycete *Allomyces arbusculus* the nuclear DNA has a buoyant density of 1.721 and mtDNA 1.710 (Ojha *et al.* 1977). This large variation in the fungal mtDNA buoyant densities reflects a high degree of heterogeneity in the DNA composition. This heterogeneity in mtDNA composition is usually expressed as G + C DNA content. For example, *Saccharomyces cerevisiae* mtDNA has a G + C content of 18% (Fonty *et al.* 1978), *Ustilago cynodontis* 33.5% (Mery-Drugeon *et al.* 1981) *U. maydis* 38% (Schildkraut *et al.* 1962) and *A. brunnescens* 26.9% (Arthur *et al.* 1982).

Occasionally, when freeze-dried cells were excessively ground, a third intermediate band was observed in the gradient with about the same or less intensity than that of the mtDNA band. The nuclear genome also contains sequences that are enriched for AT base pairs, in particular the rDNA sequences that are usually arranged in long tandem repeats. Since the nuclear genome may have over 100 copies of rDNA (Dons and Wessels 1980; Taylor *et al.* 1986), sufficient shearing of the DNA sample during preparation, may result in a large enough number of genomic rDNA fragments to generate a separate visible band between the nuclear and mtDNA bands as was observed in two of the DNA preparations. Others (Garber and Yoder 1983; Specht *et al.* 1983; Klassen *et al.* 1987) have also obtained preparations from CsCl/bisbenzimidazole isopycnic gradient centrifugations that included a third intermediate band which was determined to be rDNA. This intermediate band could be used for the rapid isolation and cloning of the rDNA repeat from *Ustilago violacea*. This procedure was used for cloning the rDNA repeat from *Pythium*, *Phytophthora* and *Apodachlya* (Klassen *et al.* 1987). Cloned rDNA has been used in hybridization experiments to obtain information on the sequence organization of rDNA genes in fungi. Thus it would provide a valuable tool for taxonomic studies in the genus *Ustilago*.

A digest of UWO-1 mtDNA with *Eco*R1 restriction enzyme resulted in ten fragments being identified on an agarose gel (Table 1). The total mitochondrial genome size of UWO-1 was estimated to be 45 ± 4 kb. The variation in size for particular fragments is mainly due to the inaccuracy involved in estimating the size of a fragment. This estimate is less than that obtained for another member of the Ustilaginales, *Ustilago cynodontis*, which has a mitochondrial genome size of 73 ± 3 kb (Mery-

Drugeon *et al.* 1981). This variation in mtDNA size among related species is not unusual among fungi. Length mutations are the most important and frequent source of variability in mtDNA in comparisons of closely related species (Bruns and Palmer 1986). For example, within the genus *Saccharomyces*, mtDNA size ranged from 23.7 kb in *S. exiguus* to 78 kb in *S. cerevisiae* (Clarke-Walker *et al.* 1983). It may also be likely that *U. violacea* and *U. cynodontis* are not very closely related. Their inclusion into the same genus has been questioned. *U. cynodontis* and *U. maydis* and numerous other species parasitize monocot plants while *U. violacea* and several other species such as *U. scabiosae* and *U. utriculosa* parasitize dicots. Investigations of sporogenesis, germination, karyogamy and cultural characteristics support the conclusion that these two groups of smut fungi are not closely related (Deml and Oberwinkler 1982). Blanz and Unseld (1987) used 5S rRNA as a molecular taxonomic character to further demonstrate that smut fungi do not form a single group but fall into two distant groups which show a high sequence diversity even within the two groups. The proper taxonomic name for *Ustilago violacea* may in fact be *Microbotryum violaceum* (Leveille 1847, *emend.* Deml and Oberwinkler 1982). Future molecular comparisons of mtDNAs from these two groups could provide additional information to help resolve this phylogenetic problem. Comparison of mtDNAs from different species would primarily involve the study of the genome structure and organization. This type of study is best accomplished with cloned mtDNA fragments which can be used to infer the number and type of mutations which produce readily observed differences (Taylor 1986).

Mitochondrial DNA was isolated and cloned in the plasmid pUC13. Of the 77 clones obtained, 18 were subsequently confirmed to contain

mitochondrial inserts (Table 3). For most isolates this confirmation consisted of demonstration that a) the cloned fragment was the same size as a known *Eco*R1 mtDNA fragment; b) radiolabeled mtDNA hybridized to the insert and c) radiolabeled insert hybridized to mtDNA but not nuclear DNA. Thus there is very little doubt that the cloned fragments were mitochondrial in origin. Except for the largest mitochondrial fragment (F1), it appears that at least one clone was obtained for each mitochondrial fragment of strain UWO-1 (Table 2). Several clones were determined to contain nuclear DNA. One of these clones apparently contains rDNA. Ribosomal DNA usually consists of long tandem repeats of over 100 copies per nuclear genome (Taylor *et al.* 1986, Kohn *et al.* 1988). This DNA consists of highly conserved sequences and is therefore useful for cross-hybridizations with DNAs from a wide variety of fungi. Furthermore, comparison of rRNAs from *E. coli* and *S. cerevisiae* indicate that prokaryotic and eukaryotic nuclear rRNAs share a small degree of homology at ribosomal protein binding sites (Planta and Raue 1988). One plasmid clone obtained in this study was about 10 kb in length. It hybridized to several restriction fragments of the nuclear DNA, showing that it probably contains sequences that are repeated in the genome. It also has slight homology to a small mitochondrial fragment of about 2.2 kb (Fig. 5). This clone may very likely contain the nuclear rDNA repeat. If the nuclear cloned fragment is indeed rDNA, then the mtDNA fragment (F8) to which the nuclear clone showed some homology might contain mitochondrial rDNA sequences.

Nine of the isolates were analyzed for RFLPs in mtDNA and the results are listed in Table 3. Fragments F3, F6 and F8 appeared to reveal no apparent polymorphisms for all the nine isolates examined.

Fragments F2 and F4 revealed large and easily recognizable polymorphisms between isolates collected in the U. K. and those of Berlin and Hungary.

The source of the RFLPs in F2 and F4 may be due to restriction site changes, insertions or deletions. The larger fragments in the Berlin and Hungary isolates' mtDNA may have resulted from the loss of a restriction site present in the mtDNA from the UWO isolates. A smaller fragment, which is observed in the UWO isolates would be part of the large fragment in the BU isolates. This seems unlikely, since the BU isolates also have the smaller 2.4 kb and 3.3 kb fragments which one would expect to have disappeared if they had been joined to the 5 kb and 7 kb fragments respectively. However, since only five fragments were analyzed, it is still possible that multiple restriction site changes or genomic rearrangements, resulting in the dislocation of restriction sites are responsible. Alternatively, additions or deletions may be directly responsible for the RFLPs. This means that the genome size may vary between BU and UWO isolates by as much as 5 kb, the sum of the difference between clones pUvm1.2 and pUvm1.4. In this case, the genome of the BU isolates could be as high as 50 kb, unless there are deletions in other restriction fragments that were not analyzed, in particular the F1 fragment which has a size of about 10 kb in isolate UWO-1. Different mitochondrial genome sizes among different isolates are not uncommon, especially since additions and deletions are the primary source of mutations in mitochondria. For example, in the analysis of four independent strains of *Schizophyllum commune*, all four strains displayed unique patterns of mtDNA RFLPs, with the genome sizes not being equal (Specht *et al.* 1983).

The mitochondrial inheritance results (Table 4) indicated that both mitochondrial types were transmitted to the progeny in the crosses that were performed. The only exception being the a_2 -nuclear type progeny of crosses involving UWO-1 and BU-3, where only the a_2 parental mitochondrial type was recovered. Biparental inheritance was also supported by the fact that in two instances, evidence for the presence of both parental mitochondrial types within a single cell was obtained (Fig. 6). Since the DNA was obtained from a culture derived from a single cell streak-purified from a bisected colony, both of the mitochondrial types must have been present in the single cell. About 40 generations (10-14 days) would have elapsed, following the initial budding-off event from the dikaryon to the growth of a single colony from the streak of the bisected colony. Cells from this colony would then be grown in liquid CM prior to DNA extraction. From the initial budding off from the dikaryon to the DNA extraction and analysis for RFLPs, well over 60 generations may be expected to have passed. In crosses of *Saccharomyces cerevisiae*, which also form mixed mitochondrial populations, most progeny had lost one of the parental mtDNA types by the 20th generation. This loss could be accounted for by the simple random effect of genetic drift (Birky 1978). Therefore, the possibility of recovering both mitochondrial types after over 60 generations in *Ustilago violacea* may be expected to be quite low, even if the progeny had initially received an equal share of mtDNA molecules from both parental types in the dikaryon. With this in mind, one might expect that the transmission of both parental mitochondrial types into hyphae may occur more frequently than could be detected with this method of analysis.

Evidence which supports this statement was obtained in the analysis of nuclear and mitochondrial genotypes from bisected colonies (Table

6). Since the hypha forms from either one or the other cell of the mated pair, the recovery of both mitochondrial types from a bisected colony showed that mitochondria are transferred through the conjugation tube. Both mitochondrial types were observed in 40% of the bisected colonies analyzed (Table 6). It is possible that the other bisected colony types had also originated from hyphae that harbored both mitochondrial types. In this instance, loss of one mitochondrial type would probably be due to genetic drift or selection. Clearly, formation of hyphae that contained both mitochondrial types occurred very frequently. Cells with mixed populations of mitochondria may, in fact, be one of the frequent results of conjugation. The formation of mixed mitochondrial populations in crosses of *U. violacea* is analogous to that observed in *Saccharomyces*. This contrasts with the biparental inheritance observed in the basidiomycetes studied to date in which mixed mtDNA populations are only present at the mating junctions, from where they are only rarely transmitted to progeny (*i.e.* fruiting bodies). The mixed mtDNA populations in these fungi probably play only an insignificant role in mtDNA inheritance.

Since mixed populations of mitochondria form in crosses of *U. violacea*, high levels of recombination would be expected as have been observed between mitochondria of *S. cerevisiae* (Fonty *et al.* 1978). However, unique recombinant mtDNA molecules which have no selective advantages conferred to them are very unlikely to be able to establish themselves within a larger population. These molecules are expected to be lost (Birky 1978). In addition, nothing is known about the physical relationship between the F2 and F4 markers. If the two fragments were located close to each other in the mitochondrial genome, the chance of their separation by recombination would be rare. Therefore, our inability

to detect recombinants does not rule out the possibility that recombination between mtDNAs of *U. violacea* does indeed occur.

The three isolates that were crossed transmitted mitochondria about equally well to offspring with no significant difference. Therefore, there appears to be no selective advantage for any of the mitochondrial types over another. Examples of situations in which one mitochondrial type has a selective advantage over another usually only involve mutant strains like the respiratory mutants of *Saccharomyces* (Ephrussi *et al.* 1955) and *Neurospora* (Griffiths and Bertrand 1984). The selective advantage conferred to the *Saccharomyces* mutants has to do with an improved replication ability of mutant mtDNAs over wild type molecules due to the presence of a greater proportion of origin of replication sequences and/or an improved segregational efficiency of the mutant mtDNA molecules into offspring (Piskur 1988). It is unlikely that one would find mitochondria that have a selective advantage over others within an interbreeding population, since the 'inferior' mitochondrial type would eventually be lost by natural selection.

Although the mitochondrial type did not appear to affect mitochondrial inheritance, the transmission of mitochondria was not random. The a₁-nuclear type progeny inherited mtDNAs equally well from both parents, while a₂-nuclear type progeny inherited mtDNAs almost exclusively from the a₂ parent (94%). The nuclear type did seem to play an important role in the transmission of mtDNAs in a₂-nuclear type progeny. Thus, although mitochondrial inheritance in *U. violacea* was by the strictest definition biparental, in a₂ cells it appeared to be almost uniparental. This very unusual aspect may in part be due to the mechanism of conjugation tube formation.

Since mating type is apparently involved in mitochondrial transmission, one possibility for a mechanism of its action may be a bias in the transfer of mitochondria through the conjugation tube. This is an attractive idea, because the development of the conjugation tube is under the control of the mating type locus. During mating, the a_2 cells form conjugation pegs which grow out as conjugation tubes toward a_1 cells and upon contact, a cytoplasmic connection is made (Poon and Day 1974). It is conceivable that this morphological asymmetry may play a role in the transmission of mitochondria. Due to the difficulty in accurately distinguishing the mating type of the pair of cells in a mated dikaryon, it was not possible to directly determine whether mitochondria could be transferred bidirectionally through the conjugation tube. An indirect method for studying mitochondrial transfer was carried out by testing how well the results of mtDNA inheritance could fit models for bidirectional or unidirectional transfer. If mitochondria are transferred bidirectionally, one would expect all four possible combinations of mitochondrial and nuclear types, a_1 (nuclear type) m_1 (mitochondria from a_1 parent), a_2m_2 , a_1m_2 and a_2m_1 to occur in equal ratios. If, on the other hand, mitochondria are transmitted only in one direction, from the a_2 to the a_1 cell and not *vice versa*, then one might expect a 1 : 3 : 3 : 1 ratio.

Both the bidirectional and the unidirectional models of mitochondrial transfer were not able to adequately explain the inheritance results obtained (Tables 9 and 10). Of the two models, the unidirectional one explains the results best ($0.02 > P > 0.01$). This type of unidirectional mechanism for mitochondrial inheritance has not been reported previously. In *Agaricus bitorquis*, where nuclear migration is normally absent, unidirectional nuclear migration occurs when a rare nuclear donating strain is paired with a nuclear recipient strain (Hintz *et*

al. 1988). It may be possible that a similar mechanism regulating mitochondrial transfer between the a_1 and a_2 cells of *U. violacea* is operating, which distinguishes mitochondrial recipient and donor strains.

An alternative explanation for the observed pattern of inheritance may be that a nuclear-encoded gene product is responsible for the selective degradation or out-replication of one parent's mtDNA in the progeny. One such mechanism has been proposed to explain uniparental mitochondrial inheritance in the cellular slime mold *Polysphodylium pallidum*. All the meiotic progeny of crosses involving closely related strains of *P. pallidum* inherited mtDNA only from the *mat2* parent (Mirfakhrai *et al.* 1990). Crosses between more distantly related strains resulted in some progeny which had *mat1* mating type. Therefore, mtDNA segregation in *P. pallidum* may not be strictly mating type dependent. The authors suggest that in the former case, the *mat2* allele is dominant to the *mat1* allele with respect to mtDNA transmission pattern, and in the latter case, that the two alleles are codominant. No mechanism for this difference has been explained.

A methylating function has been implicated in the observed bias in the mtDNA inheritance between one mating type parent and the other in interspecific crosses in the alga *Chlamydomonas* (Matagne *et al.* 1988). In *Chlamydomonas* where chloroplasts are inherited maternally and mitochondria paternally (Boynton *et al.* 1987), it has been discovered that during gametogenesis, the maternal chloroplast DNA (chlDNA) is heavily methylated while that of paternal origin is not. In the early zygote paternal chlDNA is rapidly degraded by exonucleases while the maternal chlDNA is protected by the methylation against attack (Royer and Sager 1979). Mitochondria, on the other hand, do not seem to be susceptible to the exonucleases, because the maternal mitochondria disappeared very

slowly in the zygote (Matagne *et al.* 1988). Instead, it has been suggested that mtDNA, like chlDNA in the maternal cell, is methylated and this reduces or inhibits mtDNA synthesis. As a consequence unmethylated mtDNAs of paternal origin are believed to be able to out-replicate the maternal mtDNAs, leading to the eventual elimination of the maternal mtDNAs.

An analogous system may exist in *U. violacea* although there is not yet any evidence to suggest that mtDNA of *U. violacea* is methylated. An a_1 mating type associated methylating function could explain the observed inheritance results very well. Methylated mtDNAs from a_1 parents would not be able to compete as well with unmethylated mtDNAs from a_2 parents. MtDNAs from a_1 parents were inherited only 6% of the time in the a_2 progeny. This may be due to the replication advantage a_2 mtDNAs have over methylated a_1 mtDNAs. On the other hand, a_1 mtDNAs were inherited 40% of the time in a_1 progeny of crosses. This slight statistically insignificant difference may be due to the fact that a_2 mtDNAs which find themselves in an a_1 mating type nuclear background, also become methylated like their a_1 counterparts and, therefore, have only a transient advantage in their ability to outreplicate the a_1 mtDNAs.

These mechanisms which have been invoked for regulating mitochondrial transmission in other organisms are still purely speculative for *U. violacea*. Further investigations are in order to ascertain the reasons for the correlation between mating type and the apparent aberration of biparental mitochondrial inheritance. One study may involve examining mtDNAs from a_1 and a_2 cells for methylation. Another, may involve resolving the question of bidirectional or unidirectional transfer of mitochondria through the conjugation tube. This may be achieved with a micromanipulator which can be used to place cells of

opposite mating types onto a marked surface and allowing them to conjugate and form hyphae. Once one knows from which mating type cell the hyphae are developing, one can place the mated dikaryon onto CM and allow the progeny to grow up and then analyze them for parental mitochondrial types.

Summary:

Mitochondrial inheritance in *Ustilago violacea* was determined to be biparental since both mitochondrial types were transmitted to the progeny; in a couple of cases both parental types were detected in a progeny. The ratios of parental mitochondria transmitted to the progeny, however were not equal or random in all cases: The a_1 progeny inherited mtDNA from both parents equally well; the a_2 progeny inherited mtDNA almost always from the a_2 parent (94%). The reasons for this difference are not known. No recombination of mitochondrial DNA was detected in this analysis. Knowing the mode of mitochondrial inheritance can be applied to understanding the spread of this plant pathogenic fungus and to studying taxonomic relationships within and among species.

LITERATURE CITED

- Arthur, R., Herr, F., Straus, N., Anderson, J., and P. Horgen 1982. Characterization of the genome of the cultivated mushroom, *Agaricus brunnescens*. *Exp. Mycol.* **7**: 127-132.
- Awise, J. C., and R. A. Lansman 1983. Polymorphism of mitochondrial DNA in populations of higher animals. *In* M. Nehmand, R. K. Koehn, eds., Evolution of Genes and Proteins. Sinauer, Sunderland, Mass., p. 147.
- Backer, J. S., and C. W. Birky 1985. The origin of mutant cells: mechanisms by which *Saccharomyces cerevisiae* produce cells homoplasmic for new mitochondrial mutations. *Curr. Genet.* **9**: 627-640.
- Banks, G. R. 1973. Mitochondrial DNA synthesis in permeable cells. *Nat. New Biol.* **245**: 196-199.
- Baptista-Fereira, J. E. C., Economou, A., and L. A. Casselton. 1983. Mitochondrial genetics of *Coprinus*: recombination of mitochondrial genomes. *Curr. Genet.* **7**: 405-407.
- Batcho, M., Audran, J. C., and Ch. Zambettakis 1979. Sur quelques donnees de l'evolution de l'*Ustilago violacea* (Pers.) Rouss. dans les ovules *Silene dioica* (L.). *Cl. Rev. Cytol. Biol. Veget.-Bot.* **2**: 329-346.
- Baur, E. 1909. Das wesen und die Erblichkeitsverhaltnisse der "Varietates albomarginatae hort" von Pelargonium zonale. *Z. Vererbungsbl.* **1**: 330
- Berger, S. L. 1987. Quantifying ³²P-labeled and unlabeled nucleic acids. *In* S. L. Berger and A. R. Kimmel, eds., Methods in Enzymology, v. 132, Guide to Molecular Cloning. Academic Press, New York, pp. 58-72.
- Bernardi, G., Faures, M., Piperno, G., and P. P. Slonimski. 1970. Mitochondrial DNAs from respiratory-sufficient and cytoplasmic respiratory-deficient mutant yeast. *J. Mol. Biol.* **48**: 23-42.
- Bernardi, G., Baldacci, G., Faugeron-Fonty, G., Gaillard, C., Goursot, R., Huyard, A., Mangin, M., Marotta, R., and M. de Zamoroczy. 1980. The petite mutation: excision sequences, replication origins and suppressivity. *In* A. M. Kroon and C. Saccone, eds. The organization

and expression of the mitochondrial genome. Elsevier, Amsterdam, pp. 21-33.

- Bertrand, H., Chan, B. S.-S., and A. J. E. Griffiths 1985. Insertion of a foreign nucleotide sequence into mitochondrial DNA causes senescence in *Neurospora intermedia*. *Cell* **41**: 377-384.
- Birky, C. W., Jr. 1978. Transmission genetics of mitochondria and chloroplasts. *Ann. Rev. Genet.* **12**: 471-512.
- Birky, C. W. Jr. 1983. Relaxed cellular controls and organelle heredity. *Science* **222**: 468-475.
- Birnboim, H. C., and J. Doly 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nuc. Acids Res.* **7**: 1513
- Blanc, H., and B. Dujon 1980. Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. *Proc. Natl. Acad. Sci. USA* **77**: 3942-3946.
- Blanz, P. A., and M. Unseld 1987. Ribosomal RNA as a taxonomic tool in mycology. *In* G. S. DeHoog, M. Th. Smith and A. C. M. Weijman, eds. The Expanding Realm of Yeast-Like Fungi. Elsevier, Amsterdam, pp.247-258.
- Borkhardt, B., and L. W. Olson 1983. Paternal inheritance of the mitochondrial DNA in interspecific crosses of the aquatic fungus *Allomyces*. *Curr. Genet.* **7**: 403-405.
- Borst, P., and L. A. Grivell 1978. The mitochondrial genome of yeast. *Cell* **15**: 705-723.
- Boynton, J. E., Harris, E. H., Burkhardt, B. D., Lamerson, P. M., and N. W. Gillham 1987. Transmission of mitochondrial and chloroplast genomes in crosses of *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* **84**: 2391-2395.
- Bruns, T. D., and J. D. Palmer 1986. An investigation of phylogenetic relationships within the Suilloideae using mitochondrial DNA. *Mycol. Soc. Amer. Newslett.* **37**: 21-22.
- Carnevali, F., Morpurgo, G., and G. Tecce 1969. Cytoplasmic DNA from petite colonies of *Saccharomyces cerevisiae*: A hypothesis on the nature of mutation. *Science* **163**: 1331-1333.

- Casselton, L. A., and A. Condit 1972. A mitochondrial mutant of *Coprinus lagopus*. J. Gen. Microbiol. **72**: 521-527.
- Castle, A. J., and A. W. Day 1980. Diploid derivatives of *Ustilago violacea* with altered mating type activity. I. Isolation and properties. Bot. Gaz. **141**: 85-93.
- Castle, A. J., and A. W. Day 1981. Diploid derivatives of *Ustilago violacea* with altered mating type activity. II. Polyploid segregations and mechanism of origin. Bot. Gaz. **142**: 279-285.
- Castle, A. J., and A. W. Day 1984. Isolation and identification of α -tocopherol as an inducer of the parasitic phase of *Ustilago violacea*. Phytopath. **74**: 1194-1200.
- Catterall, M. E., Baird, M. L., and E. D. Garber 1978. Genetics of *Ustilago violacea*. III. Crossing over and non-disjunction. Bot. Gaz. **139**: 266-270.
- Chambers, P., and E. Gingold 1986. A direct study of the relative synthesis of petite and grande mitochondrial DNA in zygotes from crosses involving suppressive petites of *Saccharomyces cerevisiae*. Curr. Genet. **10**: 565-571.
- Clark-Walker, G. D. 1985. Basis of diversity in mitochondrial DNAs. In T. Cavalier-Smith, ed., The Evolution of Genome Size. Wiley, New York, pp. 277-297.
- Clark-Walker, G. D., Mc Arthur, C. R., and K. S. Sriprakash 1983. Order and orientation of genetic sequences in circular mitochondrial DNA from *Sacchoromyces exiguus*. J. Mol. Evol. **19**: 331-341.
- Clark-Walker, G. D., and K. S. Sriprakash 1981. Sequence rearrangements between mitochondrial DNAs of *Torulopsis glabrata* and *Kloeckera africana* identified by hybridization with six polypeptide encoding regions from *Saccharomyces cerevisiae* mitochondrial DNA. J. Mol. Biol. **151**: 367-387.
- Correns, C. 1909. Vererbungsversuche mit blass (gelb) grünen und buntblattrigen Sippen bei *Mirabilis*, *Urtica* und *Lunaria*. Z. Vererbungsbl. **1**: 291
- Cummins, J. E., and A. W. Day 1973. Cell cycle regulation of mating type alleles in the smut fungus *Ustilago violacea*. Nature **245**: 259-260.

- Day, A. W. 1972. The isolation and identification of polyploid strains of *Ustilago violacea*. Cdn. J. Genet. Cytol. **14**: 925-932.
- Day, A. W. 1978. Chromosome transfer in dikaryons of a smut fungus. Nature **273**: 753-755.
- Day, A. W. 1979. Mating type and morphogenesis in *Ustilago violacea*. Bot. Gaz. **143**: 188-194.
- Day, A. W. and A. J. Castle 1982. The effect of host extracts on differentiation in the genus *Ustilago*. Bot. Gaz. **143**: 188-194.
- Day, A. W., Castle, A. J., and J. E. Cummins 1981. Regulation of parasitic development of the smut fungus *Ustilago violacea* by extracts from host plants. Bot. Gaz. **142**: 135-146.
- Day, A. W., and J. E. Cummins 1981. The genetics and cellular biology of sexual development in *Ustilago violacea*. In D. H. O'Day and P. A. Horgen, eds., Sexual Interactions in Eukaryotic Microbes. Academic Press, New York, pp. 379-402.
- Day, A. W., and L. L. Day 1974. The control of karyogamy in somatic cells of *Ustilago violacea*. J. Cell. Sci. **15**: 619-632.
- Day, A. W., and J. K. Jones 1968. The production and characteristics of diploids in *Ustilago violacea*. Genet. Res. **11**: 63-81.
- Day, A. W., and J. K. Jones 1969. Sexual and parasexual analysis of *Ustilago violacea*. Genet. Res. **14**: 195-221.
- Day, A. W., and J. K. Jones 1971. *p*-Flourophénylalanine-induced mitotic haploidization in *Ustilago violacea*. Genet. Res. **18**: 299-309.
- Deml, G., and F. Oberwinkler 1882. Studies in Heterobasidiomycetes. Part 24. On *Ustilago violacea* (Pers.) Rouss. from *Saponaria officinales* L. Phytopath. Z. **104**: 345-356.
- Dons, J. J. M., DeVries, O. M. H., and J. G. H. Wessels 1979. Characterization of the genome of the basidiomycete *Schizophyllum commune* Biochim. Biophys. Acta. **563**: 100-112.
- Dons, J. J. M., and J. G. H. Wessels 1980. Sequence organization of the nuclear DNA of *Schizophyllum commune*. Biochim. Biophys. Acta **607**: 385-396.

- Dujon, B. 1981. Mitochondrial genetics and functions. In J. N. Strathern, E. W. Jones, J. R. Broach, eds., The molecular biology of the yeast *Saccharomyces*. Life cycle and inheritance. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 506-508.
- Dujon, B., and P. P. Slonimski 1976. Mechanisms and rules for transmission, recombination and segregation of mitochondrial genes in *Saccharomyces cerevisiae*. In Th. Buecher, W. Neupert, W. Sebald, S. Werner, eds., Genetics and Biogenesis of chloroplast and mitochondria. Elsevier, Amsterdam, p. 393.
- Economou, A., Zolan, M. E., Pukkila, P. J., and L. A. Casselton 1986. Biased mitochondrial inheritance of optional DNA insertions following mitochondrial genome recombination in *Coprinus cinereus*. *Heredity* **57**: 127.
- Edwardson, J. R. 1970. Cytoplasmic male sterility. *Bot. Rev.* **36**: 341-420.
- Ephrussi, B., Hottinguer, H., and Y. Chimenes 1949. Action de l'acri flavine sur les levures. I. La mutation "petite colonie." *Ann. Inst. Pasteur* **76**: 351
- Ephrussi, B., Margerie-Hottinguer, H., and H. Roman 1955. Suppressiveness: A new factor in the genetic determination of the synthesis of respiratory enzymes in yeast. *Proc. Natl. Acad. Sci. USA* **41**: 1065-1071.
- Esser, K., Kuck, U., Long-Hinrichs, C., Osiewacz, H. D., Stahl, U., and P. Tudzynski 1986. Plasmids of eukaryotes. Springer, New York.
- Feinberg, A. P., and B. Vogelstein 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**: 6-13.
- Fischer, G. W., and C. S. Holton 1957. The Biology and Control of Smut Fungi. Ronald Press, New York.
- Fonty, G., Crouse, E. J., Stutz, E., and G. Bernardi 1975. The mitochondrial genome of *Euglena gracilis*. *Eur. J. Biochem.* **54**: 367-372.
- Fonty, G., Goursot, R., Wilkie, D. and G. Bernardi. 1978. The mitochondrial genome of wild-type yeast cells. *J. Mol. Biol.* **119**: 213-235.

- Fort, P., Bonhomme, E., Darlu, P., Piechaczyk, M., Jeanteur, P., and L. Thaler 1984. Clonal divergence of mitochondrial DNA versus populational evolution of nuclear genome. *Evol. Theory* **7**: 81-90.
- Garber, E. D., and A. W. Day 1985. Genetic mapping of a phytopathogenic basidiomycete, *Ustilago violacea*. *Bot. Gaz.* **146**: 449-459.
- Garber, E. D., Kerrigan, D. C., and M. R. Weil 1983. Genetics of *Ustilago violacea*. XV. Half-tetrad analysis, single selection, and double selection as mapping strategies. *Bot. Gaz.* **144**: 584-588.
- Garber, R. C., and O. C. Yoder 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal and plasmid components. *Anal. Biochem.* **135**: 410-422.
- Griffiths, A. J. F., and H. Bertrand 1984. Unstable cytoplasms in Hawaiian strains of *Neurospora intermedia*. *Curr. Genet.* **8**: 387-398.
- Grossman, L. T. and M. E. S. Hudspeth 1985. Fungal mitochondrial genomes. In J. W. Bennet, and L. L. Lasure, eds., Gene manipulations in fungi. Academic Press, London, p. 71.
- Hintz, E. A., Anderson, J. B., and P. A. Horgen 1988. Nuclear migration and mitochondrial inheritance in the mushroom *Agaricus bitorquis*. *Genetics* **119**: 35-41.
- Hintz, W. E., Mohan, M., Anderson, J. B., and P. A. Horgen 1985. The mitochondrial DNAs of *Agaricus*: Heterogeneity in *A. bitorquis* and homogeneity in *A. brunnescens*. *Curr. Genet.* **9**: 127-132.
- Holliday, R. 1961. Induced mitotic crossing-over in *Ustilago maydis*. *Genet. Res.* **2**: 231-248.
- Holliday, R. 1964. A mechanism for gene conversion in fungi. *Genet. Res.* **5**: 282-287.
- Holmes, D. S., and M. Quigley 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**: 193-197.
- Horwitz, J. P., Chua, J., Curby, R. J., Tomson, A. J., Da Rooge, B. E., Fisher, J., Mauricio, J., and I. Klundt 1964. Substrates for cytochemical demonstration of enzyme activity. I. Some substituted 3-indolyl- β -D-glycopyranosides. *J. Med. Chem.* **7**: 574.

- Hudspeth, M. E. S., Shumard, D. S., Tatti, K. M., and L. I. Grossman 1980. Rapid purification of yeast mitochondrial DNA in high yield. *Biochim. Biophys. Acta* **610**: 221-228
- Kawano, S., and T. Kuroiwa 1989. Transmission pattern of mitochondrial DNA during plasmodium formation in *Physarum polycephallum*. *J. Gen. Microbiol.* **135**: 1559-1566.
- Klassen, G. R., McNabb, S. A., and M. W. Dick 1987. Comparison of physical maps of ribosomal DNA repeating units in *Pythium*, *Phytophthora* and *Apodoachlya*. *J. Gen. Microbiol.* **133**: 2953-2959.
- Klug, W. S., and M. R. Cummings 1986. Concepts of Genetics, 2nd edn. Scott, Foresman and Co., Glenview, Ill, pp. 367-369.
- Kohn, L. M., Petsche, D. M., Bailey, S. R., Novak, L. A., and J. B. Anderson 1988. Restriction fragment length polymorphisms in nuclear and mitochondrial DNA of *Sclerotinia* species. *Am. Phytopath. Soc.* **78**: 1047-1051.
- Kuck, U., and K. Esser 1982. Genetic map of mitochondrial DNA in *Podospora anserina*. *Curr. Genet.* **5**: 143-147.
- Lehman, I. R., and C. C. Richardson 1964. The deoxyribonucleases of *E. coli*. IV. An exonuclease activity present in purified preparations of DNA polymerase. *Nature* **239**: 233-241.
- Liro, J. I. 1924. Die Ustilagineen Finnlands. *Ann. Acad. Sci. Finn., Ser. A* **17**: 30-49; 258-343.
- Manella, C. A., Goewert, R. R., and A. M. Lambowitz 1979. Characterization of variant *Neurospora crassa* mitochondrial DNAs which contain tandem reiterations. *Cell* **18**: 1197-1207.
- Maniatis, T., Fritsch, E. F., and J. Sambrook 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 86-88, 157, 250-254, 383-388, 464-468, 468, 469.
- Matagne, R. F., Rongvaux, D., and R. Loppes 1988. Transmission of mitochondrial DNA in crosses involving diploid gametes homozygous or heterozygous for the mating-type locus in *Chlamydomonas*. *Mol. Gen. Genet.* **214**: 257-262.

- May, G., and J. W. Taylor 1988. Patterns of mating and mitochondrial DNA inheritance in the agaric Basidiomycete *Coprinus cinereus*. *Genetics* **118**: 213-220.
- Mery-Dugeon, M., Crouse, E. J., Schmitt, J. M., Bohnert, H., and G. Bernardi 1981. The mitochondrial genomes of *Ustilago cynodontis* and *Acanthamoeba castellanii*. *Eur. J. Biochem.* **114**: 577-583.
- Mirfakhrai, M., Tanaka, Y., and K. Yanagisawa 1990. Evidence for mitochondrial DNA polymorphism and uniparental inheritance in the cellular slime mold *Polysphondylium pallidum*: Effect of intraspecies mating on mitochondrial DNA transmission. *Genetics* **124**: 607-613.
- Mitchell, M. B. and H. K. Mitchell. 1952. A case of "maternal" inheritance in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA* **38**: 442-449.
- Mogensen, H. L. 1988. Exclusion of male mitochondria and plastids during syngamy in barley as a basis for maternal inheritance. *Proc. Natl. Acad. Sci. USA* **85**: 2595-2597.
- Murray, M. G., and W. F. Thompson 1980. Rapid isolation of high molecular plant DNA. *Nuc. Acids Res.* **8**: 4321-4325.
- Ojha, M., Turler, H., and G. Turian 1977. Characterization of *Allomyces* genome. *Biochim. Biophys. Acta.* **478**: 377-391.
- Palmer, J. D. 1990. Contrasting modes and tempos of genome evolution in land plant organelles. *TIG* **6**: 115-120.
- Piskur, J. 1988. Transmission of yeast mitochondrial loci to progeny is reduced when nearby intergenic regions containing *ori* sequences are deleted. *Mol. Gen. Genet.* **214**: 425-432.
- Planta, R. J., and H. A. Raue 1988. Control of ribosome biogenesis in yeast. *TIG* **4**: 64-68.
- Poon, N. H., and A. W. Day 1974. Fimbriae in the fungus *Ustilago violacea*. *Nature* **250**: 648-649.
- Putrament, A., Baranowska, H., Ejchart, A., and W. Prazmo 1975. Manganese mutagenesis in yeast. III. A practical application of manganese for the induction of mitochondrial antibiotic resistant mutations. *J. Gen. Microbiol.* **62**: 265

- Rieck, A., Griffiths, A. J. F., and H. Bertrand 1982. Mitochondrial variation of *Neurospora intermedia* from nature. *Can. J. Genet. Cytol.* **24**: 741-759.
- Royer, H. D., and R. Sager 1979. Methylation of chloroplast DNA in the life cycle of *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA* **76**: 5794-5798
- Schildkraut, C. L., Marmur, J., and P. Doty 1962. Determination of the base composition of deoxyribonucleic acids from its buoyant density in CsCl. *J. Mol. Biol.* **4**: 430-440.
- Silliker, M. E. 1985. Mitochondrial inheritance in the myxomycete, *Didymium iridis*. *Mycol. Soc. Amer. Newslett.* **36**: 39-40.
- Sokal, R. R., and Rohlf, F. J. 1981. Biometry. W. H. Freeman and Company, N. Y., pp. 692-703.
- Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503-517.
- Specht, C. A., Novotny, C. P. and R. C. Ullrich 1983. Isolation and characterization of mitochondrial DNA from the basidiomycete *Schizophyllum commune*. *Exp. Mycol.* **7**: 336-343.
- Stevens, B. J. 1977. Variation in number and volume of the mitochondria in yeast according to growth conditions. A study based on serial sectioning and computer graphics reconstruction. *Biol. Cell.* **28**: 37-49.
- Strausberg, R. L., and P. S. Perlman 1978. Effect of zygote bud position on transmission of mitochondrial genes in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **163**: 131-143.
- Taylor, J. W. 1986. Fungal evolutionary biology and mitochondrial DNA. *Exp. Mycol.* **10**: 259-269.
- Taylor, J. W., Smolich, B. D., and G. May 1986. Evolution and mitochondrial DNA in *Neurospora crassa*. *Evolution* **40**: 716-739.
- Vieira, J., and J. Messing 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and regulated with synthetic universal primers. *Gene* **19**: 259

- Wallace, D. C. 1989. Mitochondrial DNA mutations and neuromuscular disease. *TIG* **5**: 9-13.
- Ward, B. L., Anderson, R. S., and A. J. Bendich 1981. The mitochondrial genome is large and variable in a family of plants (Cucurbitaceae). *Cell* **25**: 793-803.
- Weber, C. A., Hudspeth, M. E. S., Moore, G., and L. I. Grossman 1986. Analysis of the mitochondrial and nuclear genomes of two basidiomycetes, *Coprinus cinereus* and *C. sterearius*. *Curr. Genet.* **10**: 515-525.
- Wilson, A. C., Cann, R. L., Carr, S. M., George, M., Gyllensten, U. B., Helm-Bychowski, K. M., Higguchi, R. G., Palumbi, S. R., Prager, E. M., Sage, R. D., and M. Stoneking 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. *Biol. J. Linn. Soc.* **26**: 375-400.
- Zillig, H. 1921. Ueber specialisierte Formen beim Antherenbrand *Ustilago violacea* (Pers.) Fuckel. *Zentralbl. Bakteriol. Parasitenk.* **53**: 33-73.
- Zolan, M., and P. J. Pukkila 1986. Inheritance of DNA methylation in *Coprinus cinereus*. *Mol. Cell. Biol.* **6**: 195-200.